An analysis of the role of the *Schizosaccharomyces pombe* homolog of Survivin, Bir1p, in mitosis

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Dedicated to my mother

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Summary

Mitosis, the process of equal segregation of chromosomes to the two daughter cells, involves a complex series of events that are spatially and temporally coordinated to ensure that viable progeny are generated. The chromosome passenger complex, consisting of Aurora B kinase, Survivin and INCENP, is thought to mediate integration of chromosomal and cytoskeletal behavior in mitosis, based on its cellular location (Carmena and Earnshaw, 2003). The exact mechanisms by which this complex executes its various functions are in the process of being unraveled.

This study describes the analysis of the *Schizosaccharomyces pombe* homolog of Survivin, Bir1p, by utilizing methods of genetics and cell biology. *bir1*$^{+}$ is essential for cell viability. In order to gain a detailed insight into Bir1p function, conditional mutant alleles of *bir1*$^{+}$ were generated by two approaches. First, point mutations in *bir1*$^{+}$ that caused lethality at 36°C were isolated. Second, the degron approach (Dohmen *et al*., 1994) was adapted in fission yeast to generate a heat-degradable allele of *bir1*$^{+}$.

Analysis of *bir1* conditional mutants revealed that Bir1p is essential for maintaining mitotic chromosome architecture, possibly by recruiting the *S. pombe* Aurora B kinase, Ark1p, to kinetochores at the onset of mitosis. Additionally, *bir1*

mutant cells in anaphase showed the presence of 'lagging' chromosomes along the length of a bipolar spindle that failed to elongate completely. These data suggested that Bir1p might be important for proper kinetochore-microtubule interactions as well as spindle elongation during anaphase. Thus, Bir1p is important for multiple processes during mitosis.

The intracellular distribution of Bir1p was studied in detail under a variety of conditions to gain further insight into its cellular function. Bir1p is a nuclear protein that localizes to clustered centromeres in interphase cells. This protein prominently localizes to kinetochores and the spindle mid-zone during both mitotic and meiotic chromosome segregation events. The re-localization of Bir1p from kinetochores to the spindle occurs at anaphase A to B transition, and is dependent on cyclin B proteolysis, the presence of intact microtubules and the plus-end motor, Klp5p. Photo bleaching of the GFP-Bir1p signal on the kinetochore results in the absence of fluorescence on the spindle mid-zone in anaphase B, indicating that the kinetochore-localized Bir1p translocates to the spindle mid-zone. Additional photobleaching studies suggest that Bir1p undergoes minimal turnover at the spindle mid-zone. Interestingly, the behavior of this protein on the spindle mid-zone is different from that displayed by tubulin sub-units. Together, these data imply that timely re-localization of Bir1p from the kinetochores to the mid-spindle may serve to coordinate anaphase A and B. The novel dynamic behavior

of Bir1p at the spindle mid-zone suggests that the process of spindle elongation and stability is based on overlapping dynamic properties of a number of components.

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List of Abbreviations

APC	Anaphase promoting complex
bp	base pair
BSA	bovine serum albumin
DAPI	4’6, - diamino – 2 – phenylindole
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
EDTA	Ethylenediamine tetra acetic acid
EMM	Edinburgh minimal medium
GFP	green fluorescent protein
his	histidine
kb	kilo basepairs
MPF	maturation promoting factor
MTs	microtubules
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
SAC	spindle assembly checkpoint
SDS	sodium dodecyl sulphate
td	temperature degron

TE	Tris/EDTA
ts	temperature sensitive
ura	uracil
UTR	untranslated region

List of Publications

Rajagopalan, S. and Balasubramanian, M. K. (1999). S. pombe Pbh1p: an inhibitor of apoptosis domain containing protein is essential for chromosome segregation. FEBS Lett. **460**, 187-190.

Rajagopalan, S. and Balasubramanian, M. K. (2002). *Schizosaccharomyces pombe* Bir1p, a nuclear protein that localizes to kinetochores and the spindle mid-zone, is essential for chromosome condensation and spindle elongation during mitosis. Genetics **160**, 445-456.

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CHAPTER 1 – Introduction

1.1. History of cell division

A cell is the simplest unit of life. The history of cells dates back to early 17th century when Robert Hooke first observed them while examining sections of cork under a compound microscope. With advances in microscopy, it was soon realized that all plant and animal tissue was composed of aggregates of cells. These studies culminated in formulation of the cell theory by Matthias Schleiden and Theodor Schwann in 1839. Greater insight into the process of cell division came with Dumortier, Remak and Virchow, who popularized the fact that cells arose from preexisting cells by the process of fission. Key observations such as embryonic cleavages representing a series of cell divisions (Rudolf Kolliker in the 1860s), and the development of multi-cellular organisms from the conjugation of two single cells, the sperm and the egg (Pringsheim, Strasburger and Hertwig in the 1870s and 1880s), highlighted the fact that the process of cell division was fundamental to growth, development and reproduction of all life.

Even in the early days of cell biology, one of the most conspicuous and well-documented events of cell division was the process of mitosis. The German anatomist, Walther Flemming in 1882 coined the term ‘mitosis’ from the Greek word

for thread, after observing thread-like structures that spilt into two sets inside the nucleus. In 1888, Heinrich Wilhelm Waldeyer coined the term ‘chromosome’ (meaning stainable bodies) for these structures as they could be visualized by dyes. It was soon discovered that chromosomes were equally distributed to the two daughter nuclei, which led August Weismann to propose that chromosomes formed the basis of heredity. The remarkable discovery of the double-helical structure of DNA (Watson and Crick, 1953) provided deep understanding of the mechanisms of duplication and partitioning of chromosomal DNA in the cell. Such valuable insights set the stage for pioneering studies on cell division.

A sequential and coordinated set of processes that cells undergo to divide into new daughter cells is termed the cell cycle. The eukaryotic cell cycle is generally divided into four discrete phases: the synthesis phase (S) during which chromosomes are replicated; the mitotic phase (M) during which chromosome segregation and cytokinesis occur; gap phases, G1 and G2, that allow the cell to prepare for S and M phases respectively (Mitchison, 1971). Generally, the G1, S and G2 phases of the cell cycle are collectively known as interphase.

1.2. Regulation of mitosis

Landmark cell fusion experiments in mammalian cells revealed that mitosis was the most dominant state of the cell cycle, and predicted the existence of an inducer of mitosis (Rao and Johnson, 1970; Johnson and Rao, 1970). The existence of such inductive activity was proven by experiments in frog eggs that led to the discovery of the maturation promoting factor (MPF) (Masui and Markert, 1971). Fluctuations in MPF activity controlled the cell cycle, with a sharp rise in activity observed upon entry into mitosis and a drop in activity upon exit (Gerhart *et al*., 1984). The search for periodic proteins that regulated such MPF oscillations culminated in the serendipitous discovery of cyclin, a protein that was degraded at the end of each mitosis, in sea urchin eggs (Evans *et al*., 1983).

Meanwhile, elegant genetics in the fission yeast, *Schizosaccharomyces pombe* (*S. pombe*), identified a protein kinase,Cdc2p, as the master regulator of mitosis. *cdc2* loss-of-function mutations prevented entry into mitosis and gain-of-function mutations advanced mitotic entry (Nurse *et al*., 1976; Nurse and Thuriaux, 1980; Fantes, 1980). Further analysis isolated both G1 and G2 alleles of *cdc2*$^{+}$ (Nurse and Bissett, 1981). Intriguingly, the *cdc2*$^{+}$ homolog in *Saccharomyces cerevisiae* (*S. cerevisiae*), *CDC28,* was isolated first as a G1 mutant for which G2 alleles were later discovered (Hartwell *et al*., 1974; Piggott *et al*., 1982). Remarkable conservation of the evolution and function of the cell cycle machinery was demonstrated by complementation of the

temperature-sensitive fission yeast *cdc2* mutant by a human *cdc2*$^{+}$ gene as well as the budding yeast *CDC28* gene (Beach *et al*., 1982; Lee and Nurse, 1987).

The genetic and biochemical studies on regulation of mitosis were integrated when MPF was purified (Lohka *et al*., 1988). MPF was found to be a hetero-dimeric protein kinase complex of Cdc2p and cyclin B. Experiments in frog eggs clearly showed that accumulation of cyclin B induced entry into mitosis and destruction of cyclin B was essential for mitotic exit (Murray and Kirschner, 1989; Minshull *et al*., 1989; Murray *et al*., 1989). Since the Cdc2p kinase depended on cyclin B levels in order to induce mitosis, it was termed as cyclin dependent kinase (CDK). Besides availability of cyclin, CDK activity is also regulated by the phosphorylation status of its Tyr15 residue (Gould and Nurse, 1989), which is controlled by Wee1p kinase and Cdc25p phosphatase (Russell and Nurse, 1986; Russell and Nurse, 1987; Millar *et al*., 1991). The extent of regulation that controls mitotic entry and exit is not at all surprising in the context of mitosis being the penultimate and irreversible step in cell division. The end product of all this regulation is equal segregation of chromosomes and the production of two viable, genetically identical daughters.

1.3. The physical process of chromosome segregation

Once in mitosis, how does a cell ensure that its chromosomes are equally segregated? Work done in the two yeasts, *S. cerevisiae* and *S. pombe* and a variety of other higher eukaryotes have unraveled the extensive network of structural and biochemical factors including surveillance mechanisms that are essential for this event to occur with high fidelity.

1.3.1. Structural features

1.3.1.1. The chromosome

One of the main structures involved in mitosis is the chromosome. The mitotic chromosome actively participates in the process of its own segregation by possessing certain key features that enable efficient and equal separation. Some of these features are listed below.

Cohesion

A logical requirement for duplicated sets of chromosomes to be separated to opposite ends of the cell is that they remain attached to each other until they are ready for segregation. During S phase, a mechanism ensures that the replicated DNA molecules remain adhered to each other at various points along their length. These attached sets of DNA are known as sister chromatids and the ‘glue’ that holds them

together leads to sister chromatid cohesion. Cohesion between sister chromatids is maintained until the stage in mitosis when chromosome segregation occurs.

Extensive genetics and biochemistry in the budding yeast (*S. cerevisae*) have led to the discovery of a proteinaceous complex that maintains cohesion between sister chromatids. This complex, known as the cohesins, consists of four proteins, Scc1p, Scc3p, Smc1p and Smc3p. Cohesins are loaded onto chromosomes during DNA replication, and are thought to establish connections between sisters as they emerge from replication forks. Besides the core cohesin complex, a few other proteins such as Eco1p, Scc2p/Scc4p complex and Pds5p are also required to establish and maintain sister-chromatid cohesion from S-phase to mitosis (reviewed in Nasmyth, 2001).

Much insight into the structural basis of cohesion establishment stems from the molecular architecture of subunits of the cohesin complex. Smc1p and Smc3p are members of the highly conserved SMC (Structural Maintenance of Chromosomes) family of proteins (Soppa, 2001). The SMC proteins are characterized by the presence of the N and C terminal globular domains separated by long coiled-coil stretches with a globular hinge domain in the middle. In yeast, Smc1p and Smc3p are composed of an anti-parallel coiled-coil structure with the hinge domain at one end and the N- and C- termini together forming a globular head at the other end. Smc head domains belong to the ABC family of ATPases (Lowe *et al*., 2001). Smc1p and Smc3p interact

at their hinge domains to form a hetero-dimeric V-shaped structure in which one arm is composed of Smc1p and the other of Smc3p (Haering *et al*., 2002). Dimerization of the two Smc heads by ATP binding is thought to result in a functional ATPase (Hopfner *et al*., 2000). Scc1p binds to the globular heads of Smc1p and Smc3p at each end of the 'V' leading to the formation of a 'ring' that, upon ATP hydrolysis, is thought to enclose the duplicated chromatids as they emerge from the replication fork (Haering *et al*., 2002; Weitzer *et al*., 2003).

Condensation

Another important prerequisite for efficient separation of chromosomes is compaction of the amorphous, tangled mass of interphase DNA. Compaction of mitotic chromosomes occurs in two steps - firstly, the arms of sister chromatids get resolved into independent entities, followed by their shortening and thickening. Condensation prevents entanglement and provides mechanical strength to chromosomes to withstand pulling forces that occur during segregation.

Experiments in yeasts and frogs have established that a common element exists between chromosome cohesion and chromosome condensation, which is the involvement of the SMC family of proteins. Besides topoisomerase II (topoII), which has been known for its role in unwinding of DNA (Dinardo *et al*., 1984), a protein complex actively promotes chromosome resolution and compaction in mitosis. A

five-component Condensin complex was first identified in *Xenopus* and found to contain an SMC2-SMC4 heterodimer and three other non-SMC proteins (Hirano *et al*., 1997). This complex also exists in *S. pombe* (Sutani *et al*., 1999), *S. cerevisiae* (Freeman *et al*., 2000) and humans (Kimura *et al*., 2001). In *Xenopus* cell free extracts, absence of condensins results in unresolved chromatin (Hirano *et al* 1997). *S. pombe* condensin mutants (*cut3* and *cut14*) display severe defects in chromosome segregation, with unresolved masses of chromatin stretched along the length of the cell (Saka *et al*., 1994). These mutants phenotypically resemble the topoII mutants (Uemura *et al* 1987). Recent advances have shown independent localization of topoII and condensins to mitotic domains (Swedlow and Hirano, 2003).

Condensin activation is regulated at the start of mitosis by Cdc2p phosphorylation of its SMC4 subunit (Sutani *et al*., 1999). The exact mechanism of condensin-mediated condensation is currently unknown though one of the models suggests the usage of its ATPase function to stabilize large positive super-coils in a single DNA molecule (Nasmyth, 2001; Hirano, 2002). This is unlike cohesin SMCs, which are thought to modulate interactions between two different DNA molecules. Another model, very similar to the cohesin SMC model discussed previously, suggests that SMC and non-SMC subunits of the condensin complex form an ATP-dependent ring structure to enclose a coiled loop of DNA (Yoshimura *et al*., 2002). Altogether, it

is interesting to note that the SMC family of ATPases seems to be fundamental to a broad range of higher-order chromosome dynamics in mitosis.

Besides condensin-mediated compaction of DNA, phosphorylation of the serine 10 residue of histone H3 appears to be important for chromosome condensation (Wei *et al*., 1999; DeSouza *et al*., 2000). Recent evidence from *S. cerevisiae*, *S. pombe* and *Drosophila* suggests that the mitotic kinase, aurora B, may be responsible for this process (Hsu *et al*., 2000; Adams *et al*., 2001; Giet and Glover, 2001; Petersen *et al* 2001). It was hypothesized that phosphorylation of histone H3 may mediate condensation by signaling to recruit condensins onto chromosomes at mitotic onset (Wei *et al*., 1999; Giet and Glover, 2001). However, this hypothesis has been questioned by recent experiments in purified *Xenopus* egg extracts, which show that histone H3 phosphorylation is a prerequisite only for chromatid resolution (Losada *et al*., 2002) and not for chromosome compaction or for recruitment of condensins (Kimura and Hirano, 2000; Murnion *et al*., 2001; MacCallum *et al*., 2002). The exact role of this process in chromosome dynamics remains to be elucidated.

1.3.1.2. The bipolar spindle

Although chromosomes greatly facilitate the process of their own segregation, a physical apparatus is still necessary to actively separate sister chromatids to opposite

ends of the cell. A microtubule-based structure known as the bipolar spindle performs this function. Microtubules (MTs) are polymers of the tubulin protein, with each subunit made up of a heterodimer of α and β tubulin (Weisenberg *et al*., 1968). Typically, the αβ heterodimers are arranged as 13 linear protofilaments that interact laterally to form a tubular structure (Evans *et al*., 1985). At the start of mitosis, MTs are nucleated from specific structures in the cell known as centrosomes in higher eukaryotes. In yeast, this structure is known as the spindle pole body (SPB). Following nucleation, MTs grow by polymerization at both ends of the tubular structure. An inherent MT polarity is established due to the different rates of polymerization of the two ends, with the faster growing end known as the plus end and the slower growing end known as the minus end (Allen and Borisy, 1974). Underlying the function of MTs, both in mitosis and in interphase, is their tendency to exhibit dynamic instability, a property in which individual MT ends alternate between phases of polymerization and depolymerization (reviewed in Desai and Mitchison, 1997). This process is powered by the hydrolysis of GTP bound to β-tubulin (Weisenberg *et al*., 1976). The above phenomenon of microtubules governs the dynamics of the mitotic spindle and allows it to perform the mechanical action of separating sister chromatids.

Serial section electron microscopy in *S. pombe* has revealed that the spindle consists of microtubules emanating from the two spindle poles to form an anti-parallel array, with the minus ends focused and anchored at the poles and overlapping plus ends in the middle (Ding *et al*., 1993). Upon entry into mitosis, centrosomes, which duplicate once every cell cycle (Hinchcliffe and Sluder, 2001), separate into two entities and assemble a bipolar spindle. Mechanical separation of sister chromatids occurs by elongation of the mitotic spindle to which the chromosomes get attached. Spindle elongation is thought to be a result of sliding apart of the overlapping arrays of microtubules combined with continuous polymerization of tubulin at their plus ends (Cande and MacDonald, 1985; Ding *et al.*, 1993).

A number of microtubule-associated proteins (MAPs) are known to aid the process of spindle assembly and elongation. While certain MAPs increase the rate of depolymerization leading to microtubule 'catastrophe', several others are involved in stabilization of MTs by reducing catastrophe rates or increasing polymerization at plus ends (Wittmann *et al*., 2001). In addition to MAPs, other proteins such as microtubule motors regulate various aspects of spindle function. Two kinds of microtubule motor proteins exist – 1) the kinesin family of proteins that includes both plus-end and minus-end directed motors and 2) cytoplasmic dynein that is a minus-end directed motor (Goldstein and Philip, 1999; King, 2000). Experiments in the two yeasts have

shown that kinesins are important molecules in the processes of spindle assembly as well as in chromosome movement and segregation (Saunders and Hoyt, 1992; Straight *et al*., 1998; Troxell *et al*., 2001; West *et al*., 2002; Garcia *et al*., 2002). Members of the BimC family of plus-end kinesins play a role in spindle assembly and elongation by cross-linking and sliding anti-parallel microtubules in opposite directions (Enos and Morris, 1990; Hagan and Yanagida, 1992; Sharp *et al*., 1999). Aided by these mechanisms, the spindle mechanically separates the sister chromatids to cell ends.

1.3.1.3. The kinetochore

In order to be separated to the cell ends, it is essential that sister chromatids first get attached to the spindle. This attachment is facilitated by a multi-protein complex known as the kinetochore, which assembles on chromosomes. The total number of proteins that constitute the eukaryotic kinetochore complex still remains unknown, as the figure keeps increasing rapidly (Wigge and Kilmartin, 2001; Cheeseman *et al*., 2001, De Wulf *et al*., 2003). The region of DNA on which the kinetochore assembles is known as the centromere. All eukaryotes employ a varying combination of sequence-based and epigenetic means to assemble the kinetochore complex on centromeric DNA (Sullivan *et al*., 2001). Centromeres usually comprise of large stretches of highly repetitive, typically transcriptionally inactive, AT-rich

DNA. Although the centromeric sequences and their lengths are not conserved between various eukaryotes, all centromeres comprise of an inner core flanked by outer heterochromatin regions (Kniola *et al*., 2001). The inner core is built on a distinct type of chromatin, a histone H3 variant known as CENP-A (reviewed in Smith, 2002). This unique nucleosome arrangement is important for binding of various kinetochore components. The flanking outer heterochromatin region is also important for protein binding. In *S. pombe*, elegant experiments depicted the role of this region in recruiting the cohesin protein Rad21p, the fission yeast homologue of Scc1p, and hence its importance in maintaining centromeric cohesion (Bernard *et al*., 2001b; Nonaka *et al*., 2002). Together, both the inner and outer domains are essential for complete centromere function.

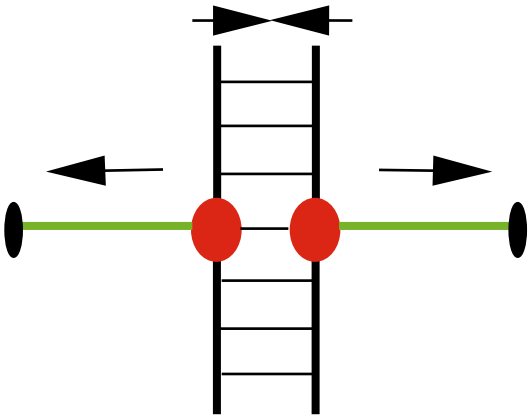
The kinetochore acts as a link between chromosomes and the spindle by serving as a platform for microtubule binding. A 'search and capture' model has been proposed in which the growing plus-ends of spindle microtubules emanating from the centrosomes, explore the surrounding intracellular space to bind to and stabilize at kinetochores. Dynamic instability of MTs greatly hastens this process of kinetochore capture (Holy and Leibler, 1994). In this context, the mitotic spindle is thus comprised of two kinds of MTs: 1) Those that are bound to and stabilized by kinetochores, known as KMTs (Kinetochore MTs) and 2) The remaining that are

stabilized by forming anti-parallel over-lapping arrays, termed as NKMTs (Non-Kinetochore MTs). Typically, only one of the attached kinetochores of the sister chromatid pair gets captured by KMTs, which causes rapid oscillation of the sisters until the other kinetochore is bound by MTs emanating from the opposite spindle pole, resulting in 'bi-orientation' of the chromosome. Subsequently, dynamic instability of KMTs results in arrangement of bi-oriented chromosomes on the spindle equator, a process termed chromosome congression (McIntosh *et al*., 2002; Biggins and Walczak, 2003). Chromosome congression precedes sister-chromatid separation.

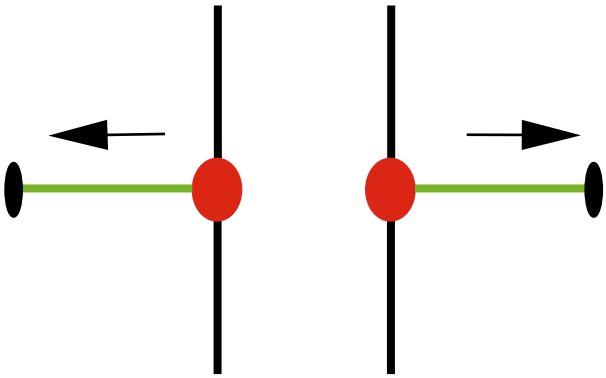
The entire process of mitosis can thus be divided into at least three distinct stages (Fig 1.1):

1) Metaphase: This stage represents a state of equilibrium in which cohesive forces that hold the condensed sister-chromatids together are balanced by the spindle MT forces that pull the sister-kinetochores apart. Studies in *S. pombe* have established that the spindle maintains a relatively constant length during this period (Nabeshima *et al*., 1998, Mallavarapu *et al*., 1999).
2) Anaphase A: Entry into anaphase A occurs upon loss of cohesion between sister-chromatids. The separated sister chromatids, bound to KMTs, move in a directed motion towards opposite spindle poles. This process has been shown to be aided by the ATP-dependent action of kinetochore-associated motors (CENP-E and Kin

Metaphase



Anaphase A



Anaphase B

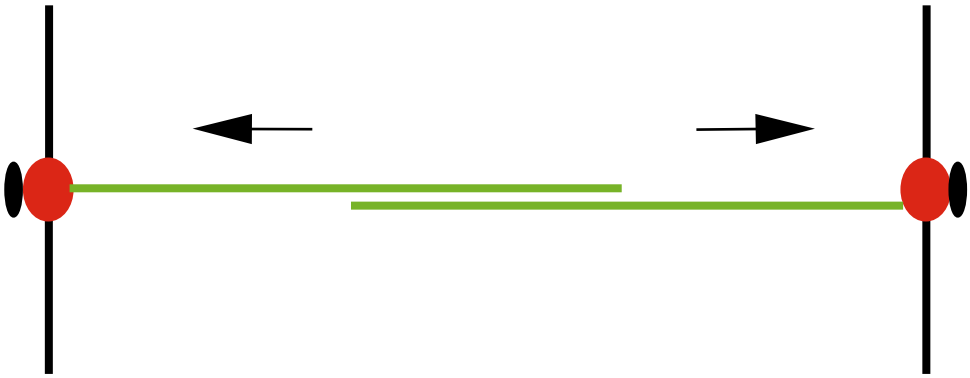


Figure. 1.1. A schematic illustration of the different stages of mitosis. Metaphase represents the stage in which chromosomes are held under a balance of forces (indicated by arrows), inward cohesive forces and outward spindle forces. Anaphase A occurs upon loss of sister-chromatid cohesion leading to movement of sister chromatids to opposite spindle poles. Spindle elongation in anaphase B initiates once chromosomes reach the poles.

I family of kinesins) that actively depolymerize KMTs (Lombillo *et al*., 1995; Desai *et al*., 1999). Slow elongation of the spindle occurs at this stage (Nabeshima *et al*., 1998, Mallavarapu *et al*., 1999).

3) Anaphase B: Anaphase A to B transition occurs when sister chromatids reach the opposite spindle poles. Rapid elongation of the spindle then occurs, by sliding of over-lapping MT arrays, to separate the spindle poles and hence the chromosomes to opposite ends of the cell (Ding *et al*., 1993; Mallavarapu *et al*., 1999).

1.3.2 Biochemical features

Key biochemical events during mitosis regulate transition from one stage to another, ensuring a coordinated and sequential course of events. Two of the main events are described below:

1.3.2.1. Loss of cohesion between sister-chromatids

Chromosomes in metaphase are held under a balance of forces, cohesive forces established by cohesins, and separating forces exerted by the mitotic spindle. Metaphase to anaphase transition depends on loss of the cohesive force component. Landmark studies in *S. cerevisiae* have established that destruction of the cohesin component, Scc1p, in metaphase is important for sister chromatid separation and hence anaphase onset (Uhlmann *et al*., 1999). A novel cysteine protease commonly

known as separase, which is encoded by *ESP1* in *S. cerevisiae* and *cut1*$^{+}$ in *S. pombe*, mediates the cleavage of Scc1p (Uhlmann *et al.*, 2000). Separase by itself is under strict regulation in order to limit its activity to metaphase. A protein known as securin, encoded by *PDS1* in *S. cerevisiae* (Ciosk *et al*., 1998), *cut2*$^{+}$ in *S. pombe* (Funabiki *et al*., 1996a & b), binds to separase and inhibits its activity.

Securin is destroyed at the metaphase to anaphase transition by ubiquitin-dependent proteolysis. A high molecular mass complex, composed of at least 11 subunits known as the APC (Anaphase Promoting Complex), functions as an E3 ubiquitin ligase to mediate covalent attachment of a multi-ubiquitin chain to securin, which is then recognized by the 26S proteasome destruction machinery (Funabiki *et al*., 1996a; Cohen-Fix *et al*., 1996). Complete activation of the APC at metaphase depends on an activator protein, Cdc20p, homologues of which are Slp1p in *S. pombe* and Fizzy in *Drosophila* (Vinstin *et al*., 1997; Kim *et al*., 1998; Dawson *et al*., 1995). Thus, a neat model has emerged in which timely destruction of securin by the APC^{Cdc20} in metaphase activates separase, which in turn destroys cohesin, thus causing sister-chromatid separation and anaphase onset.

1.3.2.2. Cyclin B destruction

Another major biochemical event that regulates mitotic progression is the proteolytic destruction of cyclin B, which results in CDK inactivation. The APC, the machinery that regulates sister-chromatid separation, also controls cyclin proteolysis (Glotzer *et al*., 1991). CDK triggers its own inactivation by promoting binding of Cdc20 to APC, resulting in cyclin B destruction by APC^{Cdc20}. Cyclin B destruction begins in metaphase and is thought to be nearly complete when anaphase begins (Clute and Pines, 1999). However, work in budding yeast suggests that CDK inactivation in metaphase may not be so clear-cut. APC^{Cdc20} initiates cyclin destruction in metaphase. However, reduction in CDK activity following cyclin proteolysis promotes the binding of another protein, Cdh1p, to APC (Vinstin *et al*., 1997). APC^{Cdh1} destroys Cdc20 and inactivates APC^{Cdc20}, and therefore assumes the task of completing the process of cyclin destruction and triggering mitotic exit (Yeong *et al*., 2000).

Cyclin proteolysis has been shown to be important for exit from mitosis (Murray *et al.,* 1989). Ubiquitin-dependent proteolysis of cyclin requires the presence of a short amino-acid sequence in its N-terminus, known as the destruction-box (Glotzer *et al*., 1991; Hershko *et al*., 1991). Expression of non-degradable versions of cyclin B, carrying deletions or mutations in the destruction-box, allowed sister-chromatid separation but prevented exit from mitosis (Murray *et al*., 1989; Holloway

et al., 1993; Surana *et al*., 1993). More recently, it was shown in *Drosophila* embryos that expression of non-degradable cyclin B caused abnormal anaphase behavior of kinetochores (Parry *et al*., 2003).

1.3.3. Surveillance mechanisms

It is important for the cell to finely regulate its biochemical events during mitosis, as they tend to be irreversible processes, once triggered. For this purpose, the cell has to ensure that all structural aspects of chromosome segregation are intact prior to the onset of biochemical events, in order to avoid mitotic catastrophy. The mechanism that monitors the status of chromosome attachment to the spindle, and delays securin and cyclin B destruction until biorientation of chromosomes is established, is called the spindle assembly checkpoint (SAC). This checkpoint is thought to monitor - 1) the presence of kinetochores unattached to MTs (Rieder *et al*., 1995), and 2) the establishment of tension at kinetochores once sister-chromatids are attached to opposite poles (Li and Nicklas, 1995; Stern and Murray, 2001). SAC function is under the control of a multi-protein complex comprising of Mad1, Mad2, Mad3, Bub1, Bub3 and Mps1, located on kinetochores. Upon structural defects, the SAC complex blocks mitotic progression by inhibition of APC^{Cdc20} activity, thereby preventing loss of sister-chromatid cohesion and cyclin destruction (reviewed in Yu,

2002). Activation of the SAC thus arrests cells in metaphase, preventing chromosome segregation until the defect is repaired.

1.4. The role of chromosomal passenger proteins in mitosis

We now know that a combination of chromosomal and cytoskeletal events regulate mitotic progression. Studies in a number of eukaryotes have identified a conserved complex of proteins known as 'chromosomal passengers' which exhibits a dramatic localization pattern during the course of chromosome segregation. Associated with kinetochores at the start of mitosis, these proteins redistribute to the spindle mid-zone in anaphase and in higher eukaryotes, they eventually localize at the cell cortex where the cleavage furrow is formed (reviewed in Carmena and Earnshaw, 2003). Based on this striking continuity in the geography of these proteins through the course of nuclear division, it was proposed that they might have a role in coordinating the chromosomal and cytoskeletal events in mitosis (Earnshaw and Bernat, 1990). Currently, the chromosomal passenger complex comprises of four proteins: 1) the mitotic kinase, Aurora B, 2) a BIR-domain containing protein, survivin, 3) the inner-centromeric protein, INCENP and 4) the mammalian telophase-disc protein, TD60.

A surge in recent papers has provided much insight into the various functions of the chromosome passenger proteins, in particular, the Aurora B kinase. Aurora B

kinase activity has been shown to be important for histone H3 phosphorylation that regulates mitotic chromosome compaction (Hsu *et al*., 2000; Adams *et al*., 2001; Giet and Glover, 2001). This kinase, along with INCENP at the kinetochore, is thought to facilitate bi-orientation of sister-chromatids by altering kinetochore-MT interactions in metaphase (Tanaka *et al*., 2002; Murata-Hori and Wang, 2002). A role for the passenger complex in maintenance of the spindle assembly checkpoint (SAC) has also been uncovered. In *S. cerevisiae*, the aurora B homolog, Ipl1p, is necessary for activation of the SAC specifically in response to lack of tension at kinetochores (Biggins and Murray, 2001). In *S. pombe*, the aurora kinase and survivin mutants, *ark1* and *bir1* respectively, fail to efficiently recruit the checkpoint protein Mad2p to the kinetochores, and thus fail to activate the SAC in response to unattached kinetochores (Petersen and Hagan, 2003).

1.5. Thesis objectives

Survivin belongs to the inhibitor of apoptosis family of proteins (IAPs) due to the presence of the BIR (Baculovirus IAP Repeat) domain (reviewed in Silke and Vaux, 2001). BIR domain-containing proteins (BIRPs), which were thought to inhibit caspases and thereby cause inhibition of apoptosis (Crook *et al*., 1993; Birnhaum *et al*., 1994), are conserved over a wide range of species. However, the anti-apoptotic

role of Survivin and its homologs began to be questioned when the *Caenorhabditis elegans* BIR-1 was shown to play a role in embryonic cytokinesis (Frasier *et al*., 1999), and the human Survivin was shown to be important for cell division (Ambrosini *et al*., 1997). Additionally, the identification of BIRPs in the two yeasts, *S. pombe* and *S. cerevisiae*, unicellular organisms that have no known apoptosis mechanisms, implied that these proteins were not specific for apoptosis, but instead might have a more conserved and basic cellular function.

The presence of a single Survivin-like protein in *S. pombe* prompted me to investigate the possible role(s) of Bir1p in cell division. I show evidence that the *S. pombe* Bir1p is essential for cell proliferation and appears to play key roles in mitotic chromosome condensation and spindle function.

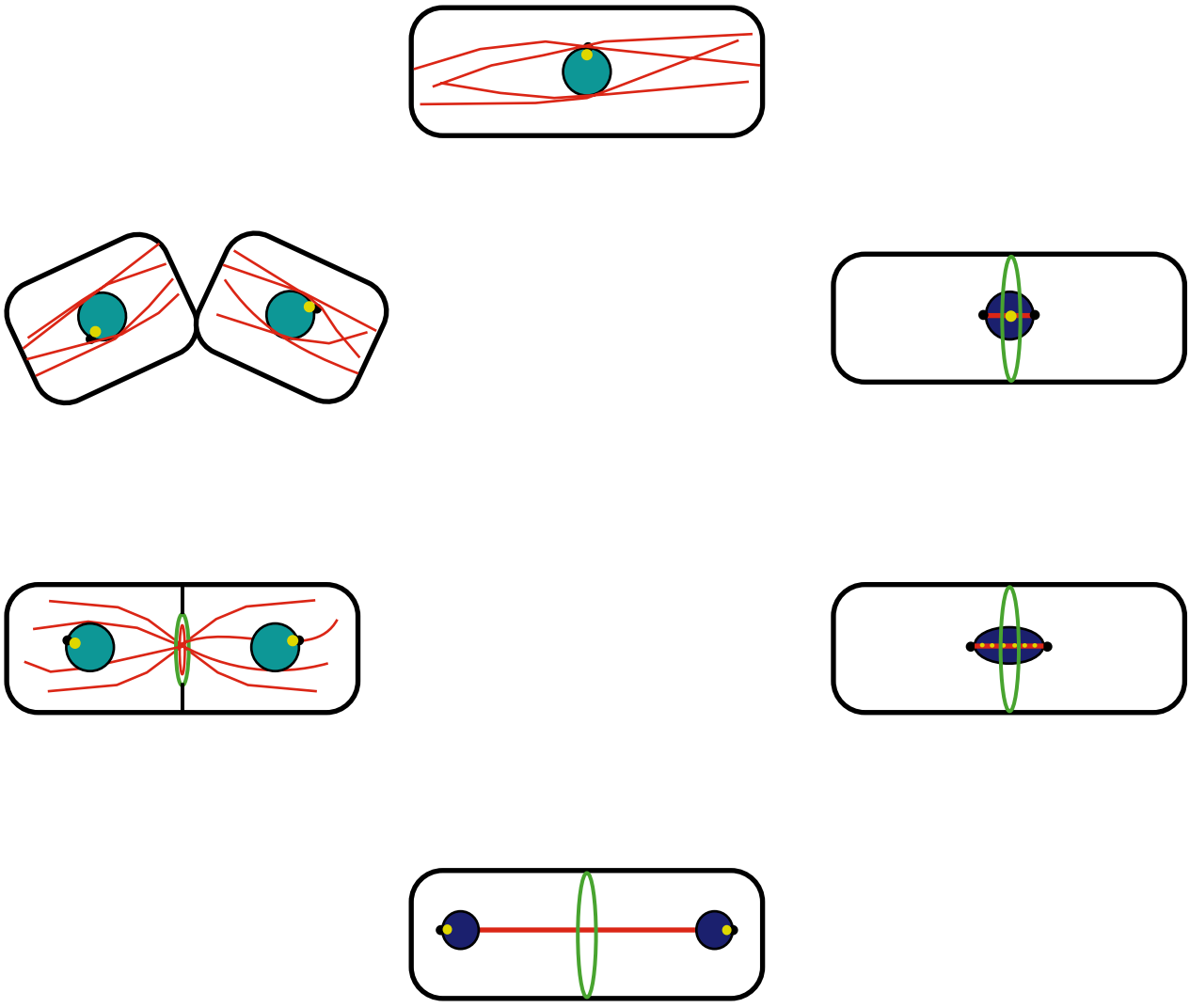
1.6. Fission yeast as a model system to analyze the mitotic role of Survivin

The fission yeast is an excellent model system to study the process of cell division, in particular mitosis. *S. pombe*, a unicelluar haploid yeast with a fully sequenced genome (Wood *et al*., 2002), is highly amenable for genetics. Although this yeast undergoes a closed mitosis in the presence of an intact nuclear envelope, the physical process of chromosome segregation is very similar to that of higher eukaryotes (Fig. 1.2). As in higher organisms, the bipolar spindle is formed only upon

mitotic entry (McCully and Robinow, 1971; Hagan and Hyams, 1988; Robinow and Hyams, 1989). Additionally, the *S. pombe* kinetochore structure highly resembles that of metazoans. Cytological studies of chromosome behavior are greatly facilitated by the presence of three chromosomes that distinctly condense at the onset of mitosis (Robinow, 1977; Hiraoka *et al*., 1984).

The ease of genetic and cytological analyses in *S. pombe* has identified a vast number of mutants that are defective in one or more stages of mitosis (Nurse *et al*., 1976; Nasmyth and Nurse, 1981; Toda *et al*., 1983; Hirano *et al*., 1986; Okhura *et al*., 1988; Yamamoto, 1988; Samejima *et al*., 1993). A distinct class of such mutants, in which nuclear division is uncoupled from cell division, displays a characteristic phenotype of the division septum 'tearing' through the unsegregated chromosomes leading to the prominent '*cut*' (cell untimely torn apart) phenotype (Hirano *et al*., 1986; Samejima *et al*., 1993). Many of the *cut* genes encode highly conserved proteins and their characterization has provided a great deal of information on the regulation of various aspects of mitosis (Yanagida, 1998).

My study will focus on the characterization of *S. pombe* Bir1p function in mitosis with the aid of conditional mutants, and analyses of its mitotic localization pattern. This study also hopes to extend our current understanding on Survivin and



condensed chromatin

interphase chromatin

actomyosin ring

centromere

SPB

microtubules

cell wall

Figure. 1.2. Mitosis in the fission yeast. Diagram shows a schematic illustration of the process of mitosis depicting the chromosomal and cytoskeletal rearrangements at each stage.

chromosomal passengers, in general, and promote further investigation and discussion on their potential roles in mitotic progression.

CHAPTER 2 - Materials And Methods

2.1 Yeast

2.1.1. Yeast strains

Genotype and origin of the *S. pombe* strains used in this study are listed in Table 1.

2.1.2. Growth and maintenance of yeast

Media used for vegetative growth of yeast strains (YES and EMM2) were as described by Moreno *et al*. (1991). YPD medium used for mating and sporulation contained 1% yeast extract (Gibco-BRL), 2% peptone (Gibco-BRL) and 2% glucose. All solid media contained 2% Bactoagar (Difco).

2.1.3. Yeast genetic and molecular methods

2.1.3.1. Mating and sporulation of yeast

Yeast matings were performed on YPD agar plates and incubated at 26°C for sporulation. Tetrads were dissected on YES plates using a Singer MSM micromanipulator (Singer Instruments, Roadwater-Watchet, Somerset, UK) to allow

germination of individual spores. Free spore analysis was performed by incubating asci in 5 ml of water containing 5 µl of NEE-154 glusulase (NEN^{TM}, Boston, USA) at 37°C for 12-13 hours. The prep was washed with 30% ethanol to remove any surviving vegetative cells. The spores were then washed thoroughly with water and plated on selective agar plates for germination. In order to observe cellular phenotypes, spores were germinated in selective EMM2 liquid media for 20 hours at 32°C.

2.1.3.2. Synchronous meiosis

A modification of the protocol described by Beach *et al* (1985) was used to allow cells to undergo synchronous meiosis. *GFP-bir1*, h^{90} cells (MBY1222) were grown to stationary phase in YES medium at 32°C. The cells were washed and inoculated at an $O.D_{595}$ of 0.5 in medium lacking both glucose and nitrogen and grown for 15 hours at 32°C. 0.2% glucose was then added to the medium, which led cells to enter meiosis synchronously. Samples were taken at 1 hour intervals to obtain cells in different stages of meiosis.

2.1.3.3. Yeast transformation

A modification of the lithium acetate protocol used by (Keeney and Boeke, 1994) was used for yeast transformation. For each transformation, 20 ml of cells at an $O.D_{595}$ of 0.5 were rinsed once with sterile water and once with 1X lithium acetate/TE-buffer (made from a 10X stock solution containing 1M lithium acetate pH 7.5, 0.1M Tris-HCl, 10mM EDTA), prior to resuspension in 80 µl of lithium acetate/TE buffer. 5-10 µg of DNA (linearised or circular) was mixed well with the cell suspension along with 50 µg of salmon sperm carrier DNA (Stratagene) and incubated at room temperature for 10 minutes. 240 µl of 70% PEG solution (8 g of PEG 4000 dissolved in 20 ml of 1X lithium acetate/TE buffer) was added to the cells and the mixture was incubated at 30°C for 30 minutes. 42 µl of dimethyl sulfoxide (DMSO) was added prior to heat-shock at 42°C and cells were plated on selective plates.

2.1.3.4. Gene disruption by homologous recombination

To delete the chromosomal copy of the *bir1*$^+$ gene, a plasmid, pCDL459 was constructed. The 5' and 3' untranslated regions (UTRs) flanking *bir1*$^+$, were cloned into pBSSK (+) such that they flanked a 1.8 kb *ura4*$^+$ gene. The plasmid was digested with *Xba*I and *Kpn*I to release the linear DNA fragment containing the *ura4*$^+$ gene flanked by the 5' and 3' UTRs of *bir1*$^+$. This linear piece of DNA was used to transform a wild-type diploid strain (MBY101/MBY104) so that the chromosomal

copy of *bir1*$^{+}$ is replaced by *ura4*$^{+}$. The diploid transformants were selected based on adenine and uracil prototrophy. The transformants were checked for correct gene replacement by polymerase chain reaction (PCR). A similar strategy was utilized to create an *ark1* null mutant, for which the marker gene used was *his3*$^{+}$ (pCDL587).

2.1.3.5. Gene tagging at the chromosomal locus

All chromosomally tagged genes were created by homologous recombination. Green fluorescent protein (GFP) and 13-Myc were the predominant tags used in this study. pJK210-based plasmids, containing the required gene fused to tags at its 5' or 3' end, were used for integration at the genomic locus as described previously (Keeney and Boeke, 1994). Putative integrants were selected based on uracil prototrophy and confirmed by PCR.

2.1.3.6. Hydroxylamine mutagenesis to isolate temperature-sensitive alleles of *bir1*$^{+}$

A modification of the hydroxylamine mutagenesis protocol described in Busby and Dreyfus (1983) was used to create a temperature-sensitive (*ts*) mutant of *bir1*$^{+}$. A 2.5 kb fragment, corresponding to 1 kb of the 5' UTR of *bir1*$^{+}$ and 1.5kb of the N-terminal region of *bir1*$^{+}$ genomic DNA, was cloned in to the integration vector, pJK210. 100 μg of this plasmid DNA was subjected to mutagenesis using 1M

hydroxylamine solution in sodium phosphate buffer, pH 6.0 at 75°C and time-points were taken at 0, 30, 60, 90 and 120 minutes after addition of hydroxylamine. The DNA from each time-point was then purified by ethanol precipitation and resuspended in 20 µl of water. 1 µl of plasmid DNA from each time-point was used to transform chemical-competent *E. coli* MC1061 cells. The extent of mutagenesis was assessed by scoring the percentage survival of ampicillin-resistant bacterial colonies. Plasmid DNA, that was mutagenised for a duration of 60 minutes, yielded a survival rate of ~50%. This DNA was then linearised with *Pst*I, which cleaved the plasmid between nucleotide positions 436-441 within the *bir1*$^{+}$ genomic DNA region, and was used to transform wild-type *S. pombe* strain (MBY192) by lithium-acetate mediated transformation. Transformants were selected at 24°C on the basis of uracil prototrophy. The colonies were replica plated to plates containing minimal agar lacking uracil and mutants were selected based on lethality at 36°C.

Five out of 600 uracil prototrophic transformants displayed a 'cut' phenotype at 36°C. Only one of the five mutants was chosen for further analysis because the other four mutants failed to display a "tight" phenotype at 36°C. The *bir1*-1 mutant was back-crossed thrice with the wild-type strain (MBY192) to ensure a 2:2 segregation of the *ts* phenotype. PCR was used to verify integration of the mutated plasmid into the chromosomal locus of *bir1*$^{+}$.

2.1.3.7. Identification of the *bir1*-1 mutation

Approximately 2 µl of *bir1*-1cells were picked from the plate and resuspended in 20 µl of sterile water. The cells were then heated at 95°C for 5 minutes prior to centrifugation. 10 µl of the supernatant liquid was used as source of genomic DNA for PCR. The first 1500 nucleotides of *bir1*-1 were amplified using primers MOH473 and MOH474. The nucleotide sequence of the amplified product from *bir1*-1 was determined by using appropriate primers and the automated PE ABI prism sequencing system and compared with wild-type *bir1*$^{+}$ sequence using the SeqMan program (Integrated DNA technologies, DNA Star) to identify the point mutation.

2.1.3.8. Construction of a thiamine-dependent *bir1*$^{+}$ shut-off strain

A linear DNA fragment, marked for uracil prototrophy, in which *bir1*$^{+}$ was under the control of the highly attenuated version of the *nmt1* promoter, *nmt1*-81 (Basi *et al*., 1993), was integrated into the chromosomal locus of *bir1*$^{+}$ by homologous recombination. The uracil prototrophs were screened for lethality when replica-plated to plates containing 5µM thiamine at 32°C. One out of ~1000 uracil prototrophs that were screened yielded a strain that was defective for growth in the presence of thiamine. This strain, denoted as P*nmt1*-81::*bir1*$^{+}$, was back-crossed to the wild-type strain (MBY192) to ensure a 2:2 segregation of the thiamine-dependent '*cut*'

phenotype and PCR was used to check integration at the *bir1*$^{+}$ chromosomal locus. The '*cut*' phenotype was apparent when cells were grown in liquid medium containing 5μM thiamine for at least two cell cycles (~5hrs) at 32°C.

2.2. *Escherichia.coli*

5.7.1. Bacterial strains

E.coli MC1061 strains were used for plasmid maintenance, amplification and rescue.

5.7.2. Plasmids

The plasmids used in this study and their origin, are listed in Table 2.

5.7.3. Growth, maintenance and selection of *E.coli*

E.coli strains were grown at 37°C in Luria-Bertani (LB) medium. Transformants were selected in LB medium containing ampicillin at a concentration of 100 μg/ml.

5.7.4. Bacterial transformation

Calcium-mediated chemical transformation method or electroporation using a Biorad™ *E.coli* pulser was used to transform *E. coli* with plasmid DNA.

5.8.**Cell biology and microscopy**

5.8.1. Reagents

To stain microtubules, mouse monoclonal anti-tubulin antibodies, TAT-1 (provided by Dr. Keith Gull, Manchester UK) were used. Rabbit polyclonal anti-GFP antibodies were obtained from Molecular Probes. Mouse monoclonal anti-*myc* antibodies were obtained from Sigma. Fluorescein isothiocyanate-conjugated secondary antibodies were obtained from Molecular Probes. 4'6, -diamidino-2-phenylindole (DAPI) was obtained from Sigma.

5.8.2. Nuclei and Septum staining

To stain the cell wall, 1 ml of exponentially growing *S. pombe* cells were fixed at 24°C for 1 minute with 3.7% formaldehyde solution and washed thrice with phosphate-buffered saline (PBS) buffer prior to the addition of calcofluor at a concentration of 5 μg/ml. To stain the nucleus, formaldehyde-fixed cells were permeabilized with PBS buffer containing 1% Triton-X 100 for 1 minute, washed thrice with PBS buffer prior to addition of DAPI at a concentration of 1 μg/ml per 20 μl of cell suspension.

2.3.3. Immunofluorescence microscopy

50 ml of exponentially growing ($O.D_{595}$ 0.2 to 0.5) yeast cells were fixed at their growth temperature with 7.4% formaldehyde solution for 10 to 12 minutes. Cells were harvested by centrifugation at 3000 rpm for 3 minutes and washed thrice with PBS buffer. The cells were then washed once with PBS containing 1.2M sorbitol before resuspension in 800 µl of the same solution. 200µl of the protoplasting enzyme mix (5 mg/ml lysing enzyme (Sigma) and 3mg/ml zymolyase (US Biological) made in PBS containing 1.2M sorbitol) was added to the cell suspension. Digestion of the cell wall was monitored by adding 9 µl of the cell suspension to 1 µl of 10% sodium dodecyl sulphate (SDS) solution on a glass slide and viewed under a light microscope. Upon sufficient protoplasting, the reaction was stopped by addition of 50 ml of PBS containing 1% Triton-X 100. The protoplasted cells were gently washed thrice with PBS before resuspension in 1 ml of PBAL blocking solution (PBS + 1% BSA, 100mM lysine hydrochloride, 50 µg/ml carbenicillin and 1mM sodium azide) for 1 hour. Primary antibodies were added at appropriate concentrations to 200 µl of cells in PBAL and incubated at room temperature overnight with shaking. Cells were then washed thrice with PBAL prior to resuspension in PBAL containing fluorochrome-conjugated secondary antibodies at a concentration of 1:200. Cells were incubated

with shaking, in the dark, for 30 minutes prior to three washes in PBAL. The cells were then resuspended in 20 µl of PBAL. 1 µl of this cell suspension was spread thinly on a glass coverslip and allowed to dry for a few minutes. 1 µl of DAPI solution was spotted on to a clean glass slide and the coverslip was inverted onto the spotted DAPI solution. All still fluorescence microcopy utilized a Leica DMLB microscope and an appropriate set of filters. Images were captured using an Optronics DEI-750T cooled CCD camera and Leica QWIN software. Image processing was done on Adobe Photoshop 5.5 and assembled using Canvas 5.0.

2.3.4. Confocal and time-lapse microscopy

For live-cell imaging of GFP-fusion proteins in *S. pombe*, 1 µl of exponentially growing cells was mounted on a borosilicate glass slide (Matsunami Trading, Japan), covered gently with a coverslip (Matsunami Trading, Japan). Time-lapse microscopy was carried out using a LEICA DM IRBE inverted microscope equipped with a LEICA N Plan 100X/1.25 oil objective and an Orca II C4742-98 CCD (charge-coupled device) camera (Hamamatsu). Images were obtained with Metamorph software and processed used Adobe Photoshop 5.5 and NIH image 1.62 (Bethesda, Maryland) and movies were assembled using Quicktime 5 (Apple, USA). Photobleaching experiments were carried out using the Laser Scanning Microscope

(LSM) 510 Axiovert inverted light microscope with Zeiss Plan Apochromat 100X/1.4 oil DIC objective, a 458, 488, 514 nm argon laser set to 100 iterations of the 488 nm He/Ar laser at 100% power. All experiments were carried out at ~24°C.

Table 1: List of strains used in this study

Strain	Genotype	Source
MBY101	*ade6*-M210 *leu1*-32 *ura4*-D18 h^-	K. Gould
MBY104	*ade6*-M216 *leu1*-32 *ura4*-D18 h^+	K. Gould
MBY175	*cdc25*-22 *ura4*-D18, h^-	K. Gould
MBY176	*cdc25*-22 *ura4*-D18, h^+	K. Gould
MBY192	*leu1*-32 *ura4*-D18 h^-	K. Gould
MBY389	*nda3*-KM311, *ura4*-D18 h^-	K. Gould
MBY527	*bir1*Δ::*ura4*$^+$/*bir1 ade6*-M210/*ade6*-M216 *leu1*-32/*leu1*-32 *ura4*-D18/*ura4*-D18 h^+/h^-	This study
MBY653	*gfp-bir1*$^+$ (*ura4*$^+$) *ura4*-D18 *leu1*-32 *ade6*-M210 h-	This study
MBY683	*gfp-bir1*$^+$ (*ura4*$^+$) *cdc25*-22	This study
MBY686	*gfp-bir1*$^+$ (*ura4*$^+$) *nda3*-KM311 *ura4*-D18	This study
MBY762	*bir1*-1 (*ura4*$^+$) *ura4*-D18 *leu1*-32 h^+	This study
MBY766	P*nmt1*-81::*bir1*$^+$ (*ura4*$^+$) *ura4*-D18 *leu1*-32 *ade6*-M210 h^-	This study
MBY776	*gfp-bir1*$^+$ (*ura4*$^+$) /*bir1*$^+$ *nda3*-KM311/*nda3*-KM311 *ade6*-M210/*ade6*-M216 *leu1*-32/*leu1*-32 *ura4*-D18/*ura4*-D18 h^+/h	This study
MBY818	*gfp-bir1*$^+$ (*ura4*$^+$) *mis6*$^+$-13*myc* (*ura4*$^+$) *ura4*-D18	This study
MBY819	*ark1*$^+$-13*myc* (*ura4*$^+$), *leu1*-32 *ura4*-D18 *ade6*-M210 h^-	This study

MBY854	*gfp-bir1*$^{+}$ *ark1*$^{+}$-13*myc* (*ura4*$^{+}$), *leu1*-32 *ura4*-D18 h^{-}	This study
MBY968	*mad2*Δ::*ura4*$^{+}$, *nda*3-KM311 *leu1*-32 *ade6*-M216 *ura4*-D18 h^{+}	D. McCollum
MBY1218	P*nmt1*-81::*bir1*$^{+}$ (*ura4*$^{+}$) *mis6*$^{+}$-13*myc* (*ura4*$^{+}$) *ura4*-D18 *leu1*-32	This study
MBY1221	P*nmt1*-81::*bir1*$^{+}$ (*ura4*$^{+}$) *nda3*-KM311	This study
MBY1222	*gfp-bir1*$^{+}$ (*ura4*$^{+}$) h^{90}	This study
MBY1334	*klp5Δ::ura4*$^{+}$ *ade6*-M216 *his3*-D1,*leu1*-32,*ura4*-D18 h^{+}	J. R. McIntosh
MBY1352	*td-gfp-bir1*$^{+}$ (*ura4*$^{+}$) *ura4*-D18 *leu1*-32 h^{-}	This study
MBY1354	*dis1*Δ::*kan*R *gfp-bir1*$^{+}$ (*ura4*$^{+}$) *ura4*-D18	This study
MBY1418	*ubr1*Δ::*ura4*$^{+}$ h^{-}	This study
MBY1419	*ubr11*Δ::*ura4*$^{+}$ h^{90}	This study
MBY1427	*dis1*Δ::*kan*R *ade6*-M210 *leu1*-32 *ura4*-D18 h^{-}	This study
MBY1486	*td-gfp-bir1*$^{+}$ (*ura4*$^{+}$) *ubr11*Δ::*ura4 ura4*-D18 *leu1*-32 h^{90}	This study
MBY1493	pREP81-*cut2*Δ*80 gfp-bir1*$^{+}$ (*ura4*$^{+}$) *leu1*-32 *ura4*-D18	This study
MBY1503	*klp5*Δ::*ura4*$^{+}$ gfp-*bir1*$^{+}$ (*ura4*$^{+}$)	This study
MBY1553	pREP41::*cdc13*Δ*81 gfp-bir1*$^{+}$ (*ura4*$^{+}$) *leu1*-32 *ura4*-D18	This study
MBY1566	*gfp-bir1*$^{+}$ (*ura4*$^{+}$) *nda3*-KM311, *mad2*Δ::*ura4*$^{+}$ *ura4*-D18	This study
MBY2182	*ark1*Δ::*his3*$^{+}$/*ark1*$^{+}$ (his3^{+}) *his3*-D1/ *his3*-D1 *ade6*-M210/*ade6*-M216 *leu1*-32/*leu1*-32 *ura4*-D18/*ura4*-D18 h^{+}/h^{-}	This study
MBY2183	P*nmt1*-81::*bir1*$^{+}$ (*ura4*$^{+}$) *ark1*$^{+}$*-gfp* (*ura4*$^{+}$) *ura4*-D18 *leu1*-32	This study

Table 2: List of plasmids used in this study

Plasmid	Vector	Enzyme sites	Insert	Source
pSK(+)	pBluescript	MCS		Stratagene
pCDL53	pJK210	MCS		Keeney and Boeke, 1994
pCDL363	pREP3X	MCS		Forsburg, 1993
pCDL392	pREP41	MCS		Basi *et al*., 1993
pCDL367	pREP81	MCS		Basi *et al*., 1993
pCDL674	pPW66R			Dohmen *et al*., 1994
pCDL459	pSK(+)	*Xba*I-*BamH*I / *Hind*III / *Sal*I-*Kpn*I	1kb 5'utr, 1.8 kb ura4$^+$, 1 kb 3'utr	This study
pCDL498	pREP3X	XhoI/SalI-BglII/BamHI	3kb bir1	This study
pCDL587	pSK(+)	*Xba*I-*BamH*I / *Hind*III / *Sal*I-*Kpn*I	1kb 5'utr (ark1), *his3*$^+$, 1 kb 3'utr (ark1)	This study
pCDL675	pJK210	*Kpn*I-*Xho*I / *Xho*I-*Bgl*II / *Bgl*II-*Not*I / *Not*I-*Sac*I	5'utr, ub-dhfr-gfp, bir1	This study
pCDL833	pREP81	*Sal*I-*BamH*I	*cut2*Δ*80*	This study
pCDL834	pREP41	*Sal*I-*BamH*I	*cdc13*Δ*81*	This study

Table 3: List of primers used in this study

Primer	Sequence
MOH403	CGTCGCGGCCGCAAACCGATAACGTCTTCTTC
MOH422	CGTCGCGGCCGCAGTAAAGGAAGA
MOH423	CGTCGCGGCCGCATTTGTATAGTTCATC
MBY473	GTCGTCAAGCTTGGCTACTTGCATAGAGTC
MBY474	GTCGTCAAGCTTGGATATGTAATGACTCTATGC
MOH635	CGTCGAGCTCAGGGTCATCATCCGGTTCCC
MOH969	CGTCCTCGAGATGCAGATT TTCGTCAAGAC
MOH970	CGTCAGATCTGGTACCGTCTTTCTTCTCG
BC17C702F	TACACTAAAAGGACTTTCTCGTTCTATATGTTATTCTTTAT TTTATCTATTTAAAAAGTTATTATTTGTAGCATGCCATGC AACAGCTATGACCCGATACCTCAACCGTGCAGGACCCTC ACTAAAGGGAAC
BC17C702R	ATCCTAATAAATAATCAAGGTGTTTGAAGCATTAAAAAA ATTAGTAGCTTAGATCACGTAAACTCCCATTGTTTAGCCT ATTGTAAAACGACGGCCGGCGGCCAAACACGGTAACTCA CTATAGGGCGAATTGG
AC15A1011F	CTTTTTTAAACCAATTTAATTATTGAAGAAAACTTTGCGG CCCAAGATCTACTTCACAGAACCTTTCCTATAATTTCATG CAACAGCTATGACCGCACGGGGAGATGATTAAGCACCCT CACTAAAGGGAAC
AC15A1011R	TCTAGTGTGATATTATCATAGACGTCGGTGACTGTGTAAT ATAAATTAGCATTAGACAATTAATAAATCGTTTTTTCTTA TTGTAAAACGACGGCCCGAGAAGGTACAGCCGTATCCAC TATCGGGCGAATTGG

CHAPTER 3 - Characterization of *bir1*$^{+}$

This chapter will focus on the functional characterization of the *S. pombe* homolog of survivin. In my study, the gene was initially named as *pbh1*$^{+}$. This gene, which has been under study by other groups as well, is also known as *bir1*$^{+}$/*cut17*$^{+}$ (Samejima *et al*., 1993; Uren *et al*., 1999; Morishita *et al*., 2001). Throughout the rest of this study, the *survivin* homolog in *S. pombe* will be referred to as *bir1*$^{+}$. This chapter describes the construction and characterization of *bir1* conditional mutants. The potential roles of Bir1p in regulating mitotic chromosome segregation are also discussed.

3.1.Identification of a BIR domain-containing protein in *S. pombe*

An *S. pombe* predicted open reading frame (ORF) with amino acid sequence similarity to the Inhibitor of Apoptosis (IAP) family of proteins was identified by the *S. pombe* genome-sequencing project (Wood *et al*., 2002). The gene encoding this ORF resides on cosmid 962 on *S. pombe* chromosome III and encodes a protein of 997 amino acids. The predicted protein sequence was used as a query to search available databases using the BLAST program (NCBI, Bethesda). Bir1p was most closely related to IAP-like proteins from human, mouse, *Drosophila* and *C. elegans*

```
HIAP-1          ------------------MNIVENSIFLSNLMKSANTFELKYDLSCELYRMSTYSTFP-- 40
MIAP-2           MDKTVSQRLGQGTLHQKLKRIMEKSTILSNWTKESEEKMKFDFSCELYRMSTYSAFP-- 57
DIAP-2          -------------------------------------MTELGMELESVRLATFGEWP-- 20
Survivin        --------------------------------MGAPTLPPAWQPFLKDHRISTFKNWPFL 28
CeBIR1          ------------------------------MAPGTKKKSDMAKFTFYKDRLMTFKNFEYD 30
SpBir1          -------------------------MKPITSSSKRRWNRFRREMCNYSKRLDTFQKKK-- 33
ScBir           -------------------------------MDGQIDKMEKRYSMTKLENRLRTFQDGVAL 30


HIAP-1          -------AGVPVSERSLARAGFYYTGVNDK---------VKCFCCG-----------LML 73
MIAP-2          -------RGVPVSERSLARAGFYYTGVNDK---------VKCFCCG-----------LML 90
DIAP-2          -------LNAPVSAEDLVANGFFATGNWLE---------AECHFCH-----------VRI 53
Survivin        -------EGCACTPERMAEAGFIHCPTENEP------DLAQCFFCF-----------KEL 64
Ce BIR1         R-----DPDAKCTSQAVAQAGFYCTGPQSG----------KCAFCN-----------KEL 64
Sp Bir1         ------WPRAKPTPETLATVGFYYNPISESNSEERL-DNVTCYMCT-----------KSF 75
Sc Bir1         EKKKLKWSFKVIPYQAMAKLGFYFDPVIDPKTSKLKKDSVRCCYCHRQTYNVRDCRSKRK 90


HIAP-1          DNWKRGDSPTEKHKKLYPS---CRFVQSLNSVNNLEATSQPTFPSSVTNSTHSLLPGTEN130
MIAP-2          DNWKQGDSPVEKHRQFYPS---CSFVQTLLSAS-LQSPSKNMSPVKSRFAHSSPLERG--144
DIAP-2          DRWEYGDQVAERHRRSSPI---CSMVLAPNHCG--------------------------- 83
Survivin        EGWEPDDDPIEEHKKHSSG---CAFLS-------------------------------- 88
Ce BIR1         D-FDPEDDPWYEHTKRDEP---CEFVR-------------------------------- 87
Sp Bir1         YDWEDDDDPLKEHITHSPS---CPWAYILS-----------------------------102
Sc Bir1         DVLETLSNIMRQHLTVTDNKQVCLLIYLRNKLLTDYSFHMGVSDWKND------------138


HIAP-1          SGYFRGSYSNSPSNPVNSRANQDFSALMRSSYHCAMNNENARLLTFQTWPLTFLSPTDLA190
MIAP-2          -----GIHSNLCSSPLNSRAVEDFSSRMDP-CSYAMSTEEARFLTYSMWPLSFLSPAELA198
DIAP-2          ---------NVPRSQESDNEGNSVVDSPESCSCPDLLLEANRLVTFKDWPNPNITPQALA134
Survivin        -----------VKKQFEELTLGEFLKLDRERAKNKIAKETN-------------------118
Ce BIR1         ----------IGKLDDSELTINDTVRLSQTAMIMTKLFEHEMMIN---------------122
Sp Bir1         -----------SKNNPNQNPQAAALTKCREQTFVDKVWPYTNRPDYHCEPSVMAASGFVY151
Sc Bir1         --------KYFSNPDDENVINLRKFTFQDNWPHSGSQNEHPLGIEKMVNAGLMRYDSSIE190


HIAP-1          KAGFYYIGP---GDRVACFACGGKLSNWEPKDNAMSEHLRHFPKCP--------------233
MIAP-2          RAGFYYIGP---GDRVACFACGGKLSNWEPKDDAMSEHRRHFPHCP--------------241
DIAP-2          KAGFYYLNR---LDHVKCVWCNGVIAKWEKNDNAFEEHKRFFPQCPRVQMGPLIEFATGK191
Survivin        ----------------------NKKKEFEETAKKVRRAIEQLAAMD--------------142
Ce BIR1         ----------------------NLSNHSSSDALFDQLKKVPNTAS--------------145
Sp Bir1         N---PTADA---KDAAHCLYCDINLHDWEPDDDPYTEHKRRRADCVFFTWKDPNSLSPTK205
Sc Bir1         GLGDPSMDKTLMNDTCYCIYCKQLLQGWSINDDPMSRHYKVSQNGN--------------236
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Figure. 3.1. Alignment of the amino acid sequences of *S. pombe* Bir1p (Sp Bir1) and the BIRPs from human (HIAP-1), human Survivin, mouse (MIAP-2), *Drosophila* (DIAP-2), *C. elegans* (Ce BIR1) and *S. cerevisiae* (Sc Bir1). Identical amino acids are indicated in red and conservative substitutions are indicated in green.

(Fig. 3.1). Amino acid sequence analysis of Bir1p revealed two imperfectly conserved BIR domains, from amino acid residue 25 to 104 and from 122 to 199. The presence of seven potential Cdc2p phosphorylation sites was also predicted.

3.2.*bir1*$^+$ is essential for cell viability

In order to analyze the effect of complete loss of function of *bir1*$^+$ in *S. pombe*, the entire coding region of *bir1*$^+$ was replaced with the *ura4*$^+$ marker by homologous recombination (described in the materials and methods section). Uracil prototrophic diploids were screened for successful replacement of one of the wild-type copies of *bir1*$^+$ by *ura4*$^+$, by polymerase chain reaction (PCR) and one strain of the genotype *bir1*::*ura*$^+$/*bir1*$^+$ was identified (MBY527). MBY527 was sporulated and tetrads were dissected on rich media. Only two spores from every tetrad formed colonies. The viable colonies were all uracil auxotrophs indicating that the haploid *bir1*::*ura*$^+$ spores were inviable. Microscopic examination revealed that the *bir1*::*ura*$^+$ spores had germinated but were arrested as single cells that had undergone a catastrophic division. This indicated that *bir1*$^+$ is essential for cell viability.

3.3.*bir1*$^+$ is essential for mitotic chromosome segregation and spindle elongation

In order to further characterize the terminal phenotype, germinated *bir1*::*ura4*$^{+}$ spores were fixed and stained with DAPI to visualize the chromosomes. *bir1* null mutants failed in chromosome segregation. In spite of abnormal nuclear division these cells underwent septation and cytokinesis normally, similar to that observed in some '*cut*' (*c*ell *u*ntimely *t*orn) mutants (Hirano *et al*., 1986). In a vast majority of cells, chromosomes appeared to be loosely organized and 'stretched' along the length of the cell and frequently cut by the division septum (Fig. 3.2).

To analyze the morphology of the mitotic spindle, germinated *bir1*::*ura4*$^{+}$ spores were fixed and stained with DAPI to visualize chromosomes and α-tubulin antibodies to visualize microtubules. Germinated *bir1* null spores displayed a typical interphase array of microtubules (data not shown). Upon entry into mitosis, these cells assembled a short spindle. In some cells, spindles of intermediate lengths were observed (Fig. 3.3). However, spindles that span the entire length of the cells, as observed in wild-type cells (Hagan and Hyams, 1988), were never detected. Septated cells displayed a typical post-anaphase array of microtubules (Fig. 3.3). The above data lead to the conclusion that *bir1*$^{+}$ is essential for mitotic chromosome segregation. It is possible that Bir1p regulates this process by effecting complete spindle elongation.

bir1*Δ::*ura4+

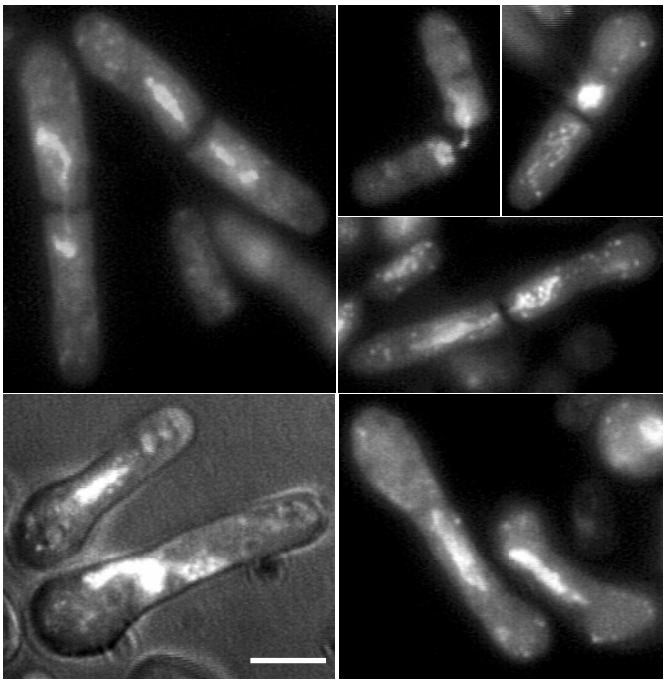


Figure. 3.2. *bir1*$^{+}$ is essential for chromosome segregation. *bir1*Δ::*ura4*$^{+}$ spores were germinated at 30°C in media lacking uracil for 20 hours, fixed with formaldehyde and stained with DAPI to visualize DNA. Scale bar = 2.2μm.

3.4.Overproduction of Bir1p causes chromosome segregation and cytokinetic defects

In order to study the effects of over-expression of Bir1p in wild-type cells, a plasmid was created in which the coding sequence of *bir1*$^{+}$ was fused downstream of the thiamine-regulated *nmt1* promoter (Basi *et al*., 1993) to produce pREP3X::*bir1*$^{+}$ (pCDL498). In the presence of thiamine, when the *nmt1* promoter is repressed, cells carrying the pREP3X::*bir1*$^{+}$ resembled wild-type cells. Upon derepression of the *nmt1* promoter in thiamine-free medium for 20 hours at 32ºC, ~15% of the cells displayed a defect in cytokinesis. These cells appeared to accumulate three or four nuclei due to inefficient cleavage of the division septum (Fig. 3.4A). In addition, ~ 4% of the cell population displayed unequal chromosome segregation and in some instances generated a *cut* phenotype (Fig. 3.4B). These data indicate that regulation of Bir1p levels in the cell is important for mitosis and cytokinesis.

3.5. Construction of conditional mutants of *bir1*

We generated conditional mutants of *bir1* in order to better understand its essential functions in mitosis in *S. pombe*. Two strategies were employed to obtain conditional mutants of *bir1* as listed below.

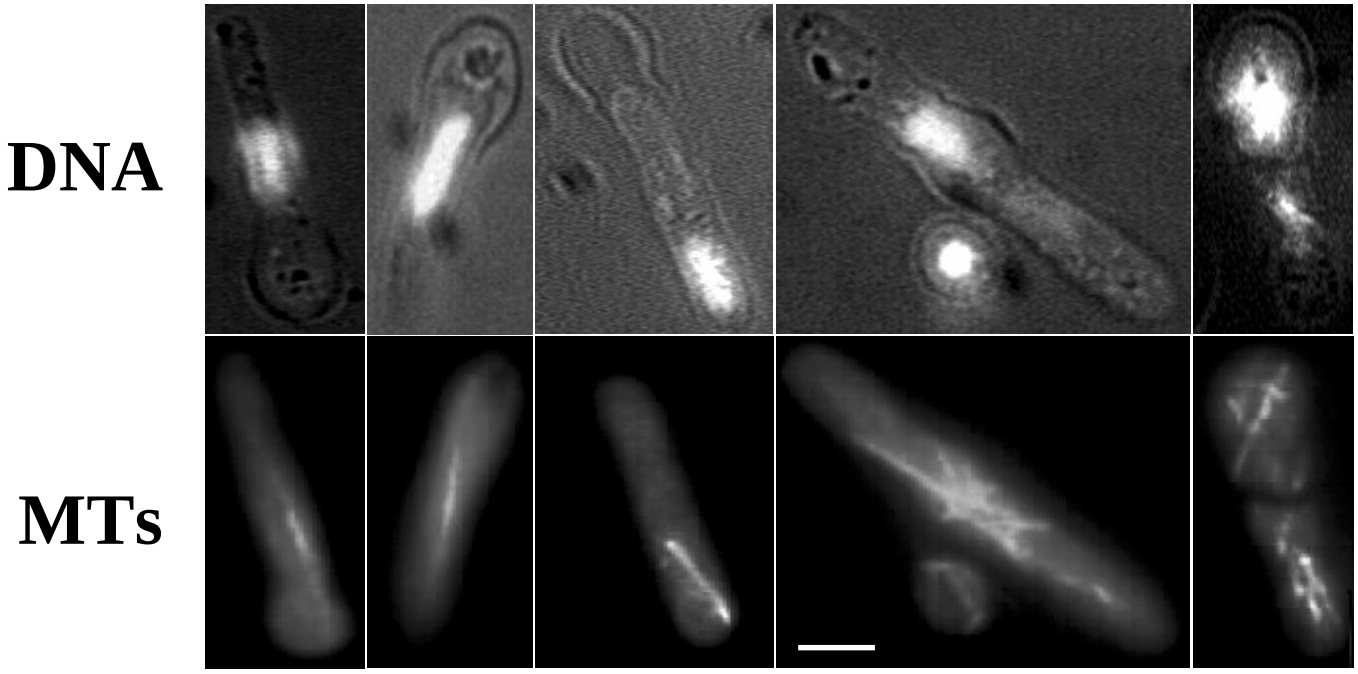

DNA
MTs

Figure. 3.3. *bir1*$^{+}$ is essential for complete spindle elongation. *bir1*Δ::*ura4*$^{+}$ spores were germinated at 30°C in media lacking uracil for 20 hours, fixed with methanol and stained with DAPI and α-tubulin antibodies to visualize DNA and microtubules (MTs) respectively. Scale bar = 2.2μm

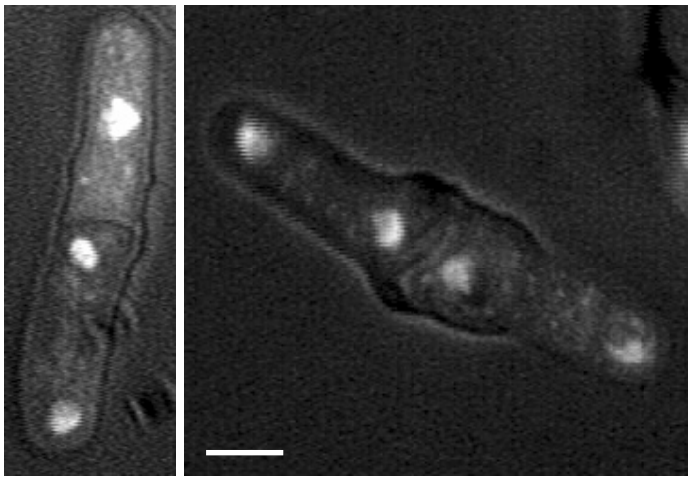
3.5.1. Temperature sensitive (ts) mutant of *bir1*

A modified method of hydroxylamine mutagenesis (described in the materials and methods section) yielded a mutant that was able to form colonies at 24°C but displayed a *cut* phenotype similar to that of the *bir1* null mutant leading to lethality at the restrictive temperature of 36°C (Fig. 3.5A). This mutant was termed *bir1*-1 (MBY762). Sequencing of the *bir1*-1 mutant revealed a single mutation that altered codon ACA to GCA resulting in the substitution of threonine at position 194 by an alanine residue. The T194A point mutation lies within the BIR domain. A heterozygous diploid strain containing the mutated copy of *bir1* in combination with *bir1*$^{+}$ did not display temperature sensitivity at 36°C indicating that the *bir1*-1 mutation is recessive.

3.5.2. Thiamine-repressible expression of Bir1p

An alternate means of conditional Bir1p expression was achieved by construction of a strain (MBY766) in which *bir1*$^{+}$ was under regulation of a thiamine-dependent promoter, P*nmt1*-81 (Basi *et al*., 1993). Screening on the basis of lethality on thiamine-containing plates yielded a strain that displayed severe chromosome segregation defects and a *cut* phenotype, similar to that of the *bir1* null mutant and *bir1*-1 mutant, upon addition of thiamine to the growth medium (Fig. 3.5B). This

A)



B)

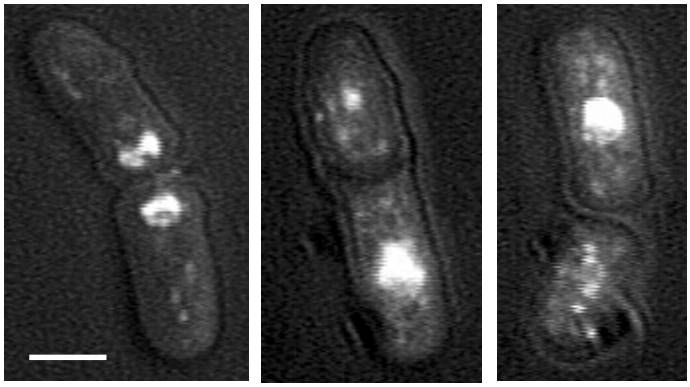


Figure. 3.4. Overproduction of Bir1p causes mitotic (A) and cytokinetic (B) defects. Wild-type cells containing pREP3X-*bir1*$^{+}$ were grown in EMM lacking thiamine at 30°C for 20 hours, fixed with formaldehyde and stained with DAPI to visualize DNA. Scale bar = 1.5μm

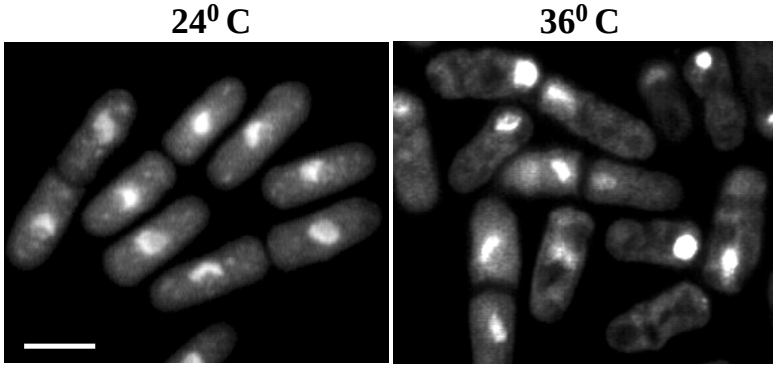
strain was denoted as P*nmt1*-81::*bir1*$^{+}$.Both conditional mutants of *bir1*, *bir1*-1 and P*nmt1*-81::*bir1*$^{+}$, were rescued by plasmid-borne wild-type *bir1*$^{+}$ (pCDL498), indicating that the observed phenotypes were due to loss of Bir1p function. These mutants were further characterized to understand Bir1p function.

3.6. Analysis of *bir1* conditional mutants

3.6.1. Bir1p is essential for mitotic chromosome condensation

In order to study the effects of Bir1p on mitosis, an asynchronously growing population of *bir1*-1cells at 24°C was shifted up to 36°C for 4 hours. A large proportion of cells undergoing mitosis had chromosomes that appeared defective in compaction and displayed a 'stretched-out' morphology along the length of the anaphase spindle (Fig. 3.6A). A similar phenotype was observed for P*nmt1*-81::*bir1*$^{+}$ grown at 32°C for 13 hours in the presence of thiamine (data not shown). In *S. pombe*, condensed chromosomes can be most clearly visualized in metaphase cells with unseparated sister chromatids (Funabiki *et al*., 1993). Therefore, in order to determine whether *bir1* mutants were defective in chromosome condensation, the effects of Bir1p depletion were studied in the β-tubulin mutant, *nda3*-KM311 (MBY389). Inactivation of tubulin in the cold sensitive *nda3*-KM311 mutant causes these cells to block at metaphase with highly condensed chromosomes due to activation of the

A)



B)

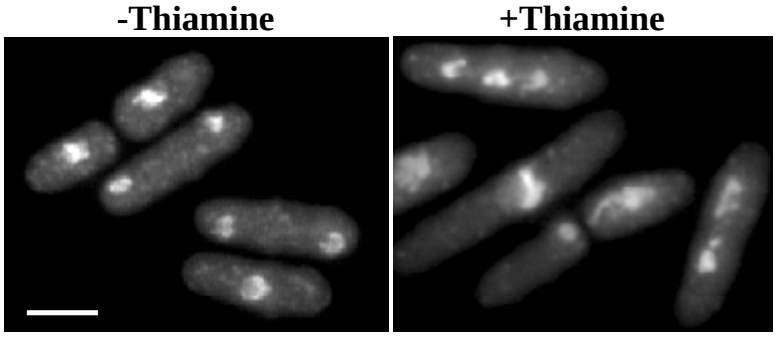


Figure. 3.5. Mitotic phenotypes of *bir1* mutants. A) *bir1*-1 cells were grown in minimal medium lacking uracil at 24°C to exponential growth phase and shifted to 36°C for 4 hours. The cells grown at both temperatures were fixed and stained with DAPI to visualize chromosomes. B) P*nmt1*-81::*bir1*$^{+}$ cells were grown to exponential growth phase at 32°C in minimal medium lacking thiamine and shifted to medium containing 5μM thiamine for 12 hours, fixed and stained with DAPI to visualize chromosomes. Scale bar = 5.7μm

spindle assembly checkpoint (Hiraoka *et al*., 1984, Rudner and Murray, 1996).The P*nmt1*-81::*bir1*$^{+}$ *nda3*-KM311 strain (MBY1221) was repressed for *bir1*$^{+}$ transcription by growth in the presence of thiamine for 5 hours. Subsequently, cold-arrest was used to enrich the population of cells blocked at metaphase. As a control, *nda3*-KM311 cells were subjected to the same growth conditions. The actomyosin ring, stained with α-Cdc4p antibodies (McCollum *et al*., 1995) was used as a marker to ensure that only mitotic cells were scored for phenotypic effects on chromosome morphology. It was observed that all *nda3*-KM311 cells, arrested at metaphase with an actomyosin ring, contained highly condensed chromosomes (Fig. 3.6B). In contrast, ~ 45% of Bir1p-depleted cells that stained for an actomyosin ring, displayed chromosomes that appeared loosely packed and uncondensed as compared to the *nda3*-KM311 mutant control (Fig. 3.6C). These data suggested that Bir1p might be essential for some aspect of initiation and possibly maintenance of chromosome condensation at the onset of mitosis.

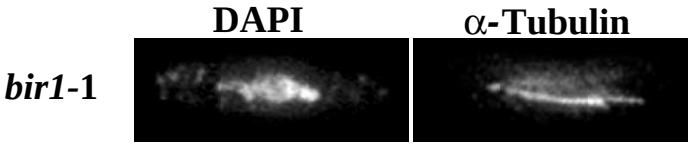
3.6.2. Bir1p is essential for mitotic localization of the *S. pombe* aurora kinase B homolog, Ark1p.

3.6.2.1. Construction of the *ark1* null mutant

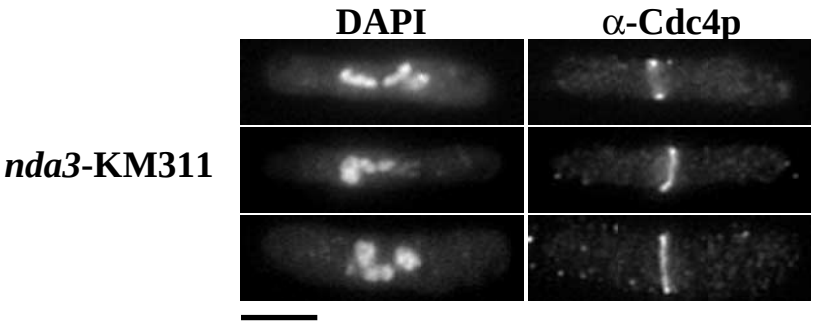
Studies in a number of eukaryotes have suggested that Bir1p along with the mitotic kinase, aurora B, and the inner centromeric protein, INCENP, form a chromosome passenger complex that is thought to be important for a variety of functions during mitosis and cytokinesis (Carmena and Earnshaw, 2003). The Aurora B kinase homologue in *S. cerevisiae*, Ipl1p, has been shown to be important for chromosome condensation via its role in histone H3 phosphorylation (Hsu *et al*., 2000). In this context, there existed a possibility that the apparent chromosome condensation defects in cells depleted of Bir1p were a result of impaired Aurora B kinase function. A protein kinase related to Aurora B has been identified by the *S. pombe* genome sequencing project (Wood *et al*., 2002). This protein was designated as Aim1p (**a**urora and **I**pl1 related **m**itotic kinase) in this study. Others named it as Ark1p (**a**urora **r**elated **k**inase) (Morishita *et al*., 2001). This protein will henceforth be referred to as Ark1p in this manuscript.

To test the physiological role of Ark1p in *S. pombe*, a diploid strain was constructed in which one copy of the *ark1*$^+$ gene was replaced by the *his3*$^+$ marker gene (MBY2182). Tetrad analysis of the haploid spores from this strain revealed that *ark1*$^+$ is essential for cell viability. Further examination of the germinated spores revealed that *ark1*$^+$ was important for chromosome segregation. ***ark1Δ*** cells displayed chromosomes that appeared ‘stretched’ on the anaphase spindle which were eventually

A)


DAPI
α-Tubulin
bir1-1

B)


DAPI
α-Cdc4p
nda3-KM311

C)

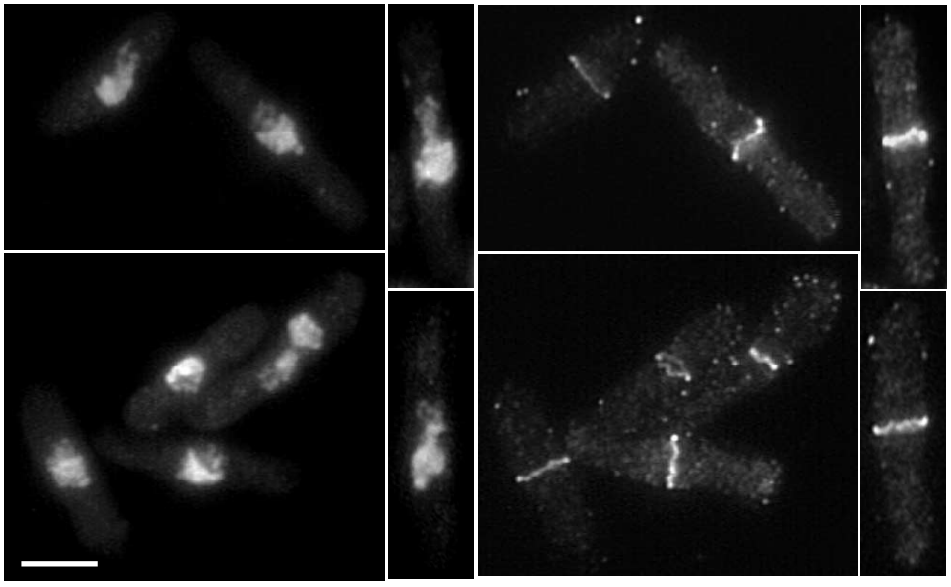

P*nmt1*-81::*bir1*,
nda3-KM311

Figure. 3.6. Bir1p is essential for chromosome condensation. A) *bir1*-1 cells were grown to exponential growth phase at 24°C and shifted to 36°C for 4 hours. Cells were fixed with DAPI and α-tubulin antibodies to visualize chromosomes and microtubules respectively. B) *nda3*-KM311 cells were grown to exponential growth phase at 32°C, shifted to 18°C for 6 hours, fixed and stained with DAPI to visualize chromosomes, and with α-Cdc4p to visualize the actomyosin ring. C) P*nmt1*-81::*bir1*$^{+}$ *nda3*-KM311 cells were grown at 32°C to exponential growth phase in minimal medium lacking thiamine and transferred to medium containing 5μM thiamine for 5 hours. Cells were then shifted to 18°C for 6 hours, fixed and stained with DAPI to to visualize chromosomes, and with α-Cdc4p to visualize the actomyosin ring. Scale bar = 4.7μm

'cut' by the division septum (Fig. 3.7). The loosely-packed arrangement of chromosomes on the anaphase spindle in the *ark1*Δ cells largely resembled that of the *bir1* null mutant.

3.6.2.2. Ark1p co-localizes with Bir1p in mitosis

To study the intracellular localization of *S. pombe* Aurora B kinase, a strain expressing Myc epitope - tagged Ark1p under the control of its native promoter was constructed (MBY819). Immuno-fluorescence studies with anti-myc and anti-tubulin antibodies revealed that Ark1p was detected at spot-like structures in early mitotic cells and at the spindle mid-zone in anaphase cells (data not shown). Interestingly, wild-type cells expressing a fusion protein of GFP-Bir1p (construction of GFP-Bir1p is explained in Chapter 5, section 5.1) revealed that Bir1p localizes to kinetochores in metaphase cells and moves to the mid-spindle in anaphase (detailed description in Chapter 5, section 5.1). In order to compare Ark1p localization with that of Bir1p in mitotic cells, GFP-Bir1p was expressed in MBY819 (MBY854). Co-stainings with anti-Myc and anti-GFP antibodies in MBY854 revealed that Ark1p co-localized with Bir1p at kinetochores and the spindle mid-zone (Fig. 3.8A). Interestingly, in a small proportion of early mitotic cells that displayed prominent kinetochore staining of Bir1p, Ark1p was not detected on any structure (Fig. 3.8A i). This observation

***ark1*Δ::*ura*4+**

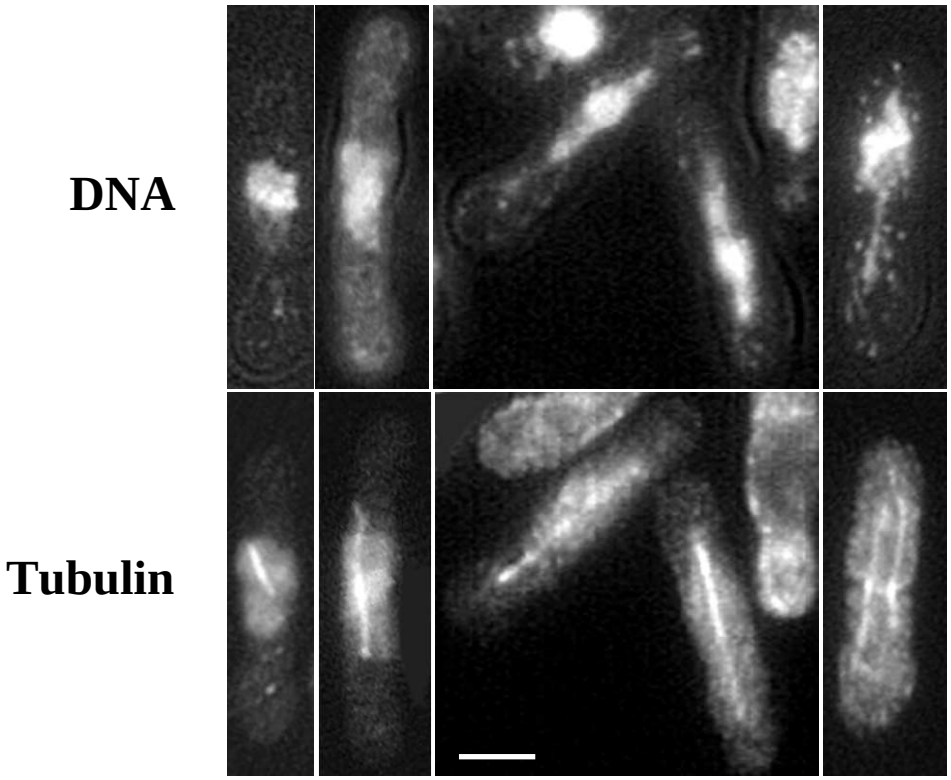


Figure. 3.7. Ark1p is required for chromosome segregation. *ark1*Δ::*his3*$^{+}$ spores were germinated in medium lacking histidine for 20 hours, fixed and stained with DAPI to visualize chromosomes and α-tubulin antibodies to visualize microtubules. Scale bar = 3.8μm.

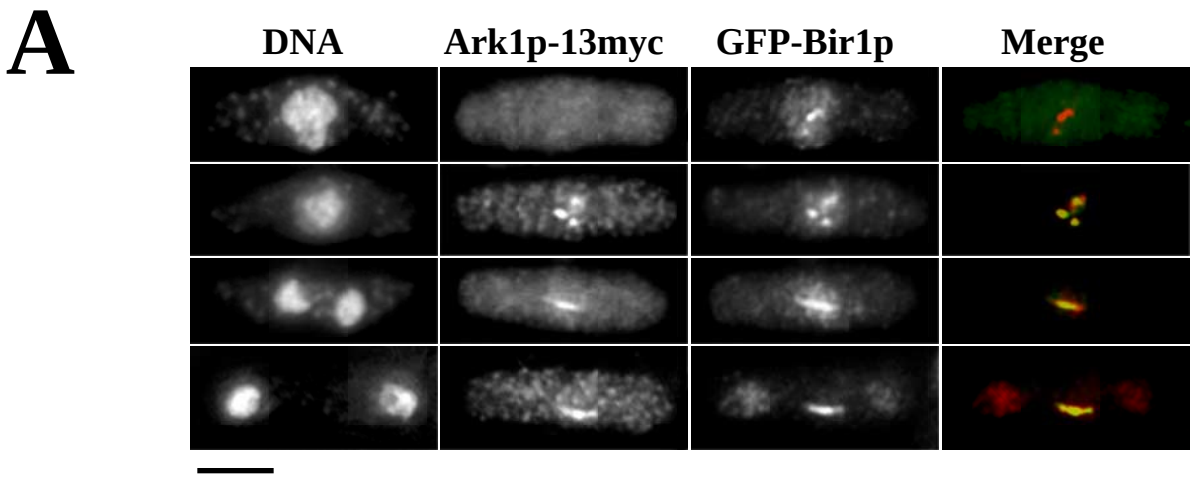
suggests that the localization of Bir1p to kinetochores may precede that of Ark1p in mitosis.

3.6.2.3 Ark1p fails to localize in mitotic cells lacking Bir1p

In the context of previous observations, it was interesting to test whether Bir1p was required for the mitotic localization of Ark1p. For this purpose, a P*nmt1*-81::*bir1*$^{+}$ strain, expressing GFP-tagged Ark1p under the control of its native promoter, was analyzed (MBY2183). This strain was grown in the presence of thiamine for 13 hours at 32ºC in order to shut-off Bir1p expression. A control culture was grown at similar conditions in the absence of thiamine. In cells depleted of Bir1p, it was observed that both kinetochore and spindle mid-zone localization of Ark1p was completely abolished, as opposed to control (Fig. 3.8B). This result clearly demonstrated the dependency of Ark1p localization on Bir1p during mitosis.

3.6.3. Cells depleted of Bir1p display chromosomes that lag on the anaphase spindle

One of the other prominent mitotic defects exhibited by cells depleted of Bir1p, was the presence of discrete chromosome masses trailing along the length of the anaphase spindle (Fig. 3.9A). In order to ascertain that these were indeed lagging chromosomes, Mis6p, a kinetochore marker (Saitoh *et al*., 1997), C-terminally tagged


A
DNA
Ark1p-13myc
GFP-Bir1p
Merge

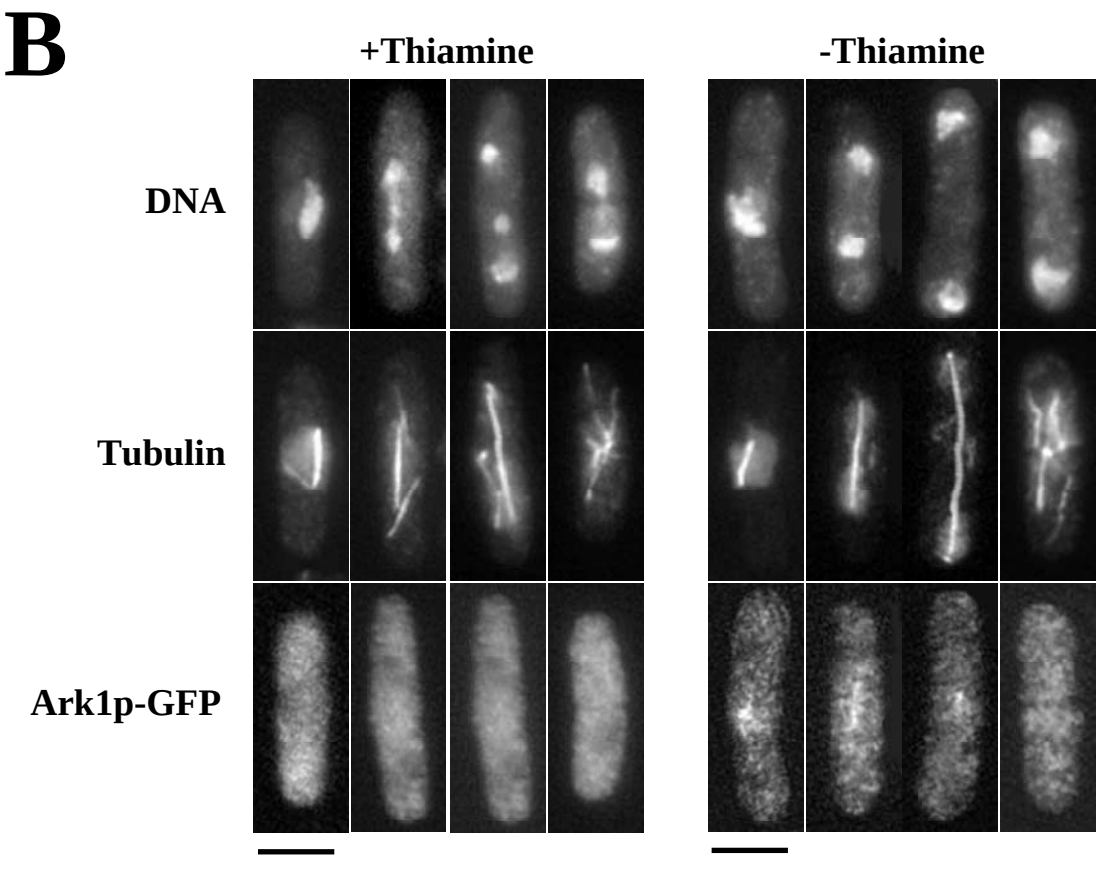

B
+Thiamine
-Thiamine
DNA
Tubulin
Ark1p-GFP

Figure. 3.8. Bir1p function is required for mitotic localization of Ark1p. A) Cells expressing GFP-Bir1p and Ark1p-13Myc were grown to exponential phase, fixed and stained with DAPI to visualize chromosomes, α-GFP to visualize GFP-Bir1p and α-myc to visualize Ark1p-13Myc. Merge represents an overlap of Ark1p (green) and Bir1p (red) staining. B) P*nmt1*-81::*bir1*$^{+}$ cells were grown at 32°C to exponential growth phase in minimal medium lacking thiamine, shifted to medium containing 5μM thiamine for 12 hours, fixed and stained with DAPI to visualize chromosomes, α-GFP to visualize Ark1p-GFP and α-tubulin to visualize microtubules. Scale bar = 4.7μm.

with 13-Myc at its chromosomal locus, was introduced into the P*nmt1*-81::*bir1*$^{+}$ strain. This strain (MBY1218) was shifted to thiamine-containing medium to turn-off *bir1*$^{+}$ transcription, fixed and stained with α-Myc antibodies to detect kinetochores. Approximately 69% of mitotic cells (138/200 mitotic cells scored) depleted of Bir1p displayed "lagging chromosomes" with kinetochores detected at several points along the length of the anaphase spindle. The kinetochores co-localized with the nuclear material indicating that the trailing pieces of DNA were indeed entire chromatids (Fig. 3.9B). In many cases, more than three kinetochores were observed along the spindle indicating that it was individual chromatids rather than non-disjoined chromosomes that lagged in anaphase B (Fig. 3.9B rows i, ii & iv). It was also observed that in certain cells, the distribution of kinetochores to both the spindle poles was unequal, indicative of asymmetric segregation of chromatids to the ends of the spindle (Fig. 3.9B row i). These observations indicated that Bir1p was required for synchronous and symmetric segregation of chromosomes in anaphase.

3.6.4. Bir1p is important for complete anaphase spindle elongation

Spore germination studies of the *bir1*Δ mutant had shown that these cells were defective in anaphase spindle elongation. To further investigate this phenotype, P*nmt1*-81::*bir1*$^{+}$ cells, grown in thiamine-containing medium, were fixed and stained

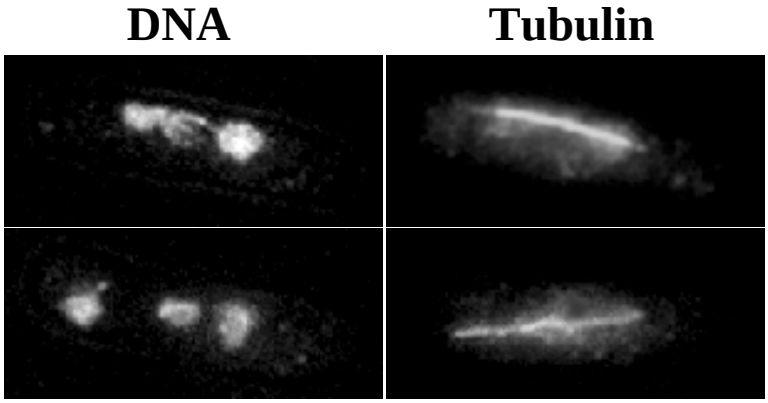
to visualize chromosomes and the mitotic spindle. Control cells grown in the absence of thiamine displayed normal chromosome segregation to the two cell ends with the aid of the anaphase B spindle that spanned the entire length of the cell (Fig. 3.10A i-v). In contrast, cells depleted for Bir1p displayed severe chromosome segregation defects as described earlier. Additionally, these cells, though capable of assembling a mitotic spindle and initiating elongation in anaphase to some extent, were unable to completely elongate the spindle to effectively separate chromosomes to opposite ends of the cell (Fig. 3.10A vi-x). A percentage count of spindle lengths depicted an ~80% reduction in the number of fully elongated spindles in mitotic cells that lacked Bir1p, as compared to control (Fig. 3.10B). This reduction was compensated by an increase in the number of intermediate- length spindles. These observations suggested that Bir1p was important for complete elongation of the anaphase B spindle.

3.7.Discussion

3.7.1. Chromosome condensation

This study has shown that *bir1* mutants display defects in mitotic chromosome architecture. Whereas wild-type cells display highly condensed chromosomes at the ends of the mitotic spindle, condensed chromosome masses are not observed in mitotic

A



B

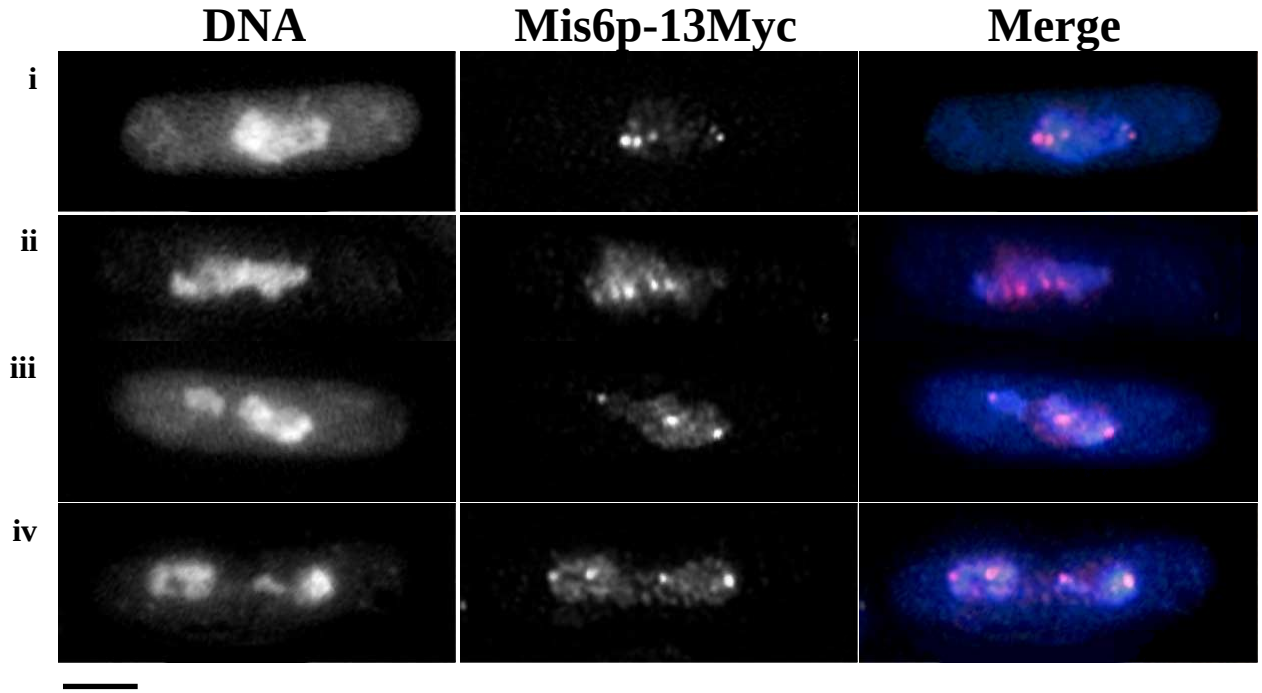
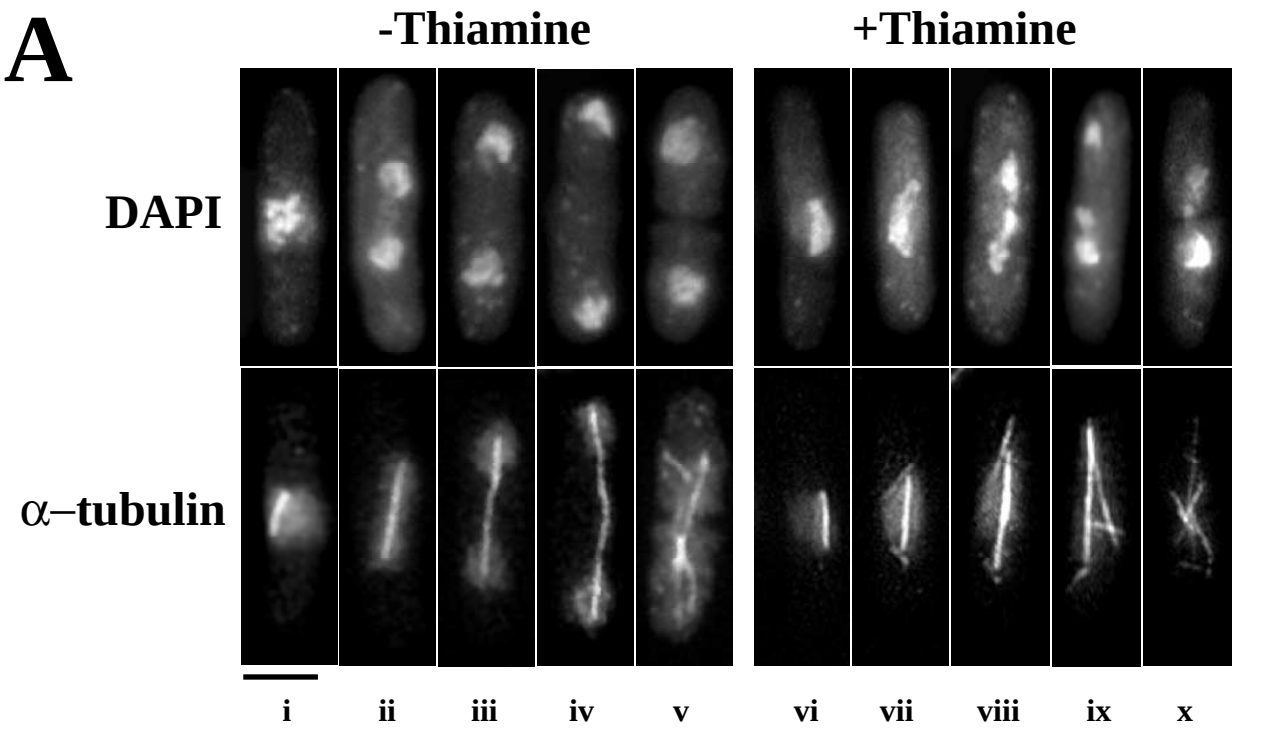


Figure. 3.9. Cells depleted of Bir1p display chromosomes that lag on the anaphase spindle. A) P*nmt1*-81::*bir1*$^{+}$ cells were grown at 32°C to exponential growth phase in minimal medium lacking thiamine, shifted to medium containing 5μM thiamine for 12 hours, fixed and stained with DAPI to visualize chromosomes and α-tubulin to visualize microtubules. B) A P*nmt1*-81::*bir1*$^{+}$ strain expressing Mis6p-13Myc was grown at 32°C to exponential growth phase in minimal medium lacking thiamine, shifted to medium containing 5μM thiamine for 12 hours, fixed and stained with DAPI to visualize chromosomes and α-myc to visualize Mis6p-stained kinetochores. Merge indicates an overlap of DAPI (blue) and α-myc (red)-stained images. Row i demonstrates unequal segregation of kinetochores. Scale bar = 3.4μm.

bir1 mutant cells. Furthermore, β-tubulin mutants are known to arrest at mitosis with condensed chromosomes (Hiraoka *et al*., 1984). Interestingly, β-tubulin mutants lacking Bir1p do not display condensed chromatin. These data strongly suggest a role for Bir1p in mitotic chromosome condensation, possibly both in establishment as well as maintenance. An independent study (Morishita *et al*., 2001) has also reported the function of Bir1p in chromosome condensation. Thus, it appears that Bir1p is important for chromosome architecture during mitosis. In future, more rigorous time-lapse studies are required to firmly establish whether Bir1p is essential for both establishment as well as maintenance of chromosome condensation or for maintenance only.

Aurora B kinase has been implicated in mediating chromosome compaction through phosphorylation of the serine 10 residue in histone H3 (Hsu *et al*., 2000). It has been proposed that histone H3 phosphorylation might serve as a signal to recruit the Condensin complex on chromosomes (Wei *et al*., 1999; Giet and Glover, 2001). The Condensin complex, which is essential for mitotic chromosome condensation (Hirano *et al*., 1997), is conserved in *S. pombe*, and consists of five proteins - Cut3p, Cut14p and Cnd1-3p (Sutani *et al*., 1999). This study has shown that the presence of functional Bir1p is essential for the recruitment of Ark1p to kinetochores as well as to the spindle mid-zone in mitosis. It has also been shown that both *bir1* and *ark1*


A
-Thiamine
+Thiamine
DAPI
α–tubulin
i
ii
iii
iv
v
vi
vii
viii
ix
x

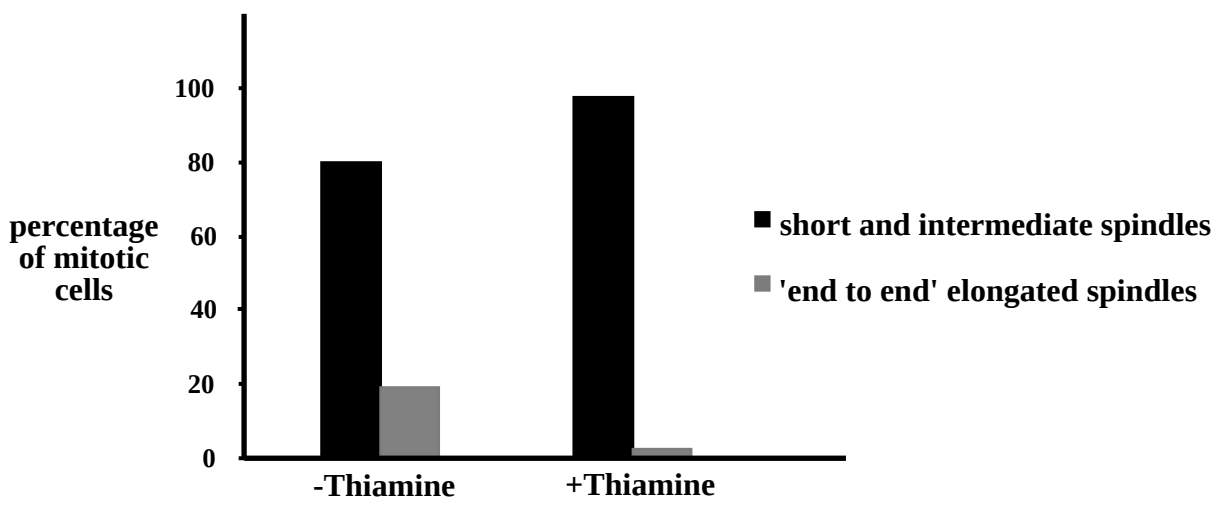

B
percentage of mitotic cells
100
80
60
40
20
0
-Thiamine
+Thiamine
short and intermediate spindles
'end to end' elongated spindles

Figure. 3.10. Bir1p function is required for complete spindle elongation in mitosis. A) P*nmt1*-81::*bir1*$^{+}$ cells were grown at 32°C to exponential growth phase in minimal medium lacking thiamine, shifted to medium containing 5μM thiamine for 12 hours, fixed and stained with DAPI to visualize chromosomes and α-tubulin to visualize microtubules. i-v and vi-x represent different stages of mitosis in cells grown in the absence and presence of thiamine respectively. i and vi represent cells with short spindles. ii, iii, vii, viii and ix are examples of cells with intermediate-length spindles. iv represents an example of an end-to-end elongated spindle. A comparison of iv and ix points out the defect in spindle elongation in cells depleted of Bir1p. B) P*nmt1*-81::*bir1*$^{+}$ cells grown in the absence and presence of thiamine (as described in A) were counted for the number of cells containing short, intermediate, and end-to-end elongated spindles. The graph represents a percentage comparison of cells with short and intermediate-length spindles (black) and end-to-end elongated spindles (grey). Scale bar = 4.1μm.

mutants appear to be phenotypically similar with respect to defects in mitotic chromosome architecture. It is possible that Bir1p may physically recruit Ark1p to the kinetochores, which may eventually lead to loading of the Condensin machinery onto chromosomes. This hypothesis is supported by recent evidence, which shows that *S. pombe ark1* mutants fail to phosphorylate histone H3 and recruit the Condensin component, Cut3p (Petersen and Hagan, 2003). Another study has shown that a *bir1* temperature sensitive mutant, *cut17*-275, is impaired for Cut3p and Cnd2p recruitment to chromosomes in mitosis (Morishita *et al*., 2001).

Interestingly, the mitotic chromosome architecture of *S. pombe* Condensin mutants, in which the bulk of chromatin remains in the middle of an elongated spindle leading to the formation of ϕ-shaped chromosomes (Saka *et al*., 1994; Sutani *et al.*, 1999), is quite distinct from that of *bir1* and *ark1* mutants. Hence, the structural defects in *bir1* and *ark1* mutants may not be solely attributed to the lack of condensins. It has been shown in *Xenopus* extracts that histone H3 phosphorylation by Aurora B kinase is important for sister-chromatid resolution (Losada *et al.*, 2002). In this study, the presence of six kinetochores in anaphase in the *bir1* mutant indicated the occurrence of centromeric disjunction in mitosis. However, the status of arm-cohesion, which is indicative of sister-chromatid resolution, needs to be investigated in this mutant. Thus, a variety of events that require Aurora B kinase activity in mitosis

may regulate chromosome compaction and resolution. Bir1p may play an essential role in this process via recruitment of Ark1p in a spatial and temporal manner.

3.7.2. The chromosome passenger complex

It has been previously proposed that the BIR-domain containing protein, Survivin, is part of a chromosome passenger complex together with the mitotic kinase, Aurora B, and the inner centromere protein, INCENP. In higher eukaryotes, this complex is thought to move from centromeres in early mitosis to the mid-spindle in anaphase and eventually to the mid-body and cleavage furrow during cytokinesis. Based on its cellular location, this complex is thought to perform various functions in mitosis and cytokinesis. In fission yeast, the Survivin homolog, Bir1p, and the Aurora B kinase homolog, Ark1p, localize to kinetochores and the spindle mid-zone during mitosis as in mammalian cells. This study has shown that the *S. pombe* Bir1p and Ark1p are important for a number of functions that ensure fidelity of chromosome segregation in mitosis. It has also been shown that Ark1p co-localizes with Bir1p at kinetochores in metaphase and at the spindle mid-zone in anaphase and its mitotic localization is dependent on the presence of functional Bir1p. Future biochemical studies should address whether Bir1p and Ark1p function as a complex to execute their various functions in mitosis.

Interestingly, unlike in higher organisms, no distinct staining of either Ark1p or Bir1p was observed at the division site. Although both *bir1* and *ark1* null mutants showed severe defects in chromosome segregation, they were not obviously impaired for cytokinesis. In fact, cell division occurred in the absence of nuclear division leading to a *cut* phenotype in these mutants. Although a possible role for the passenger complex in coordinating mitosis with cell division cannot be ruled out, it appears that these proteins in *S. pombe* may not be directly involved in the process of cytokinesis. A similar status seems to exist for these proteins in *S. cerevisiae* (Uren *et al*., 1999; Francisco and Chan, 1994). It is possible that the chromosome passenger complex may have acquired additional functions in cytokinesis through the course of evolution.

3.7.3. Chromosome segregation

This study has shown that cells depleted of Bir1p display aberrant chromosome behavior in anaphase. Kinetochore staining of chromosomes, which appeared 'stretched-out', revealed that they were 'lagging' on the anaphase spindle. The presence of more than three kinetochores indicated that individual, dis-joined sister-chromatids lagged in anaphase. Additionally, asymmetric segregation of chromatids to the spindle poles was also apparent.

'Lagging' chromosome behavior has previously been observed in fission yeast heterochromatin mutants, such as *clr4*, *csp1* and *swi6*, which are disrupted for kinetochore function (Allshire *et al*., 1995; Ekwall *et al*., 1999). Such behavior is typically thought to arise due to impaired kinetochore-MT interactions. This study has shown that Bir1p localizes to kinetochores in mitosis. In an independent study, Morishita *et al* (2001) have shown that Bir1p binds to outer repetitive regions of the fission yeast centromere. Interestingly, the heterochromatin-organizing proteins, Swi6p and Chp1p have been shown to bind to the outer centromeric regions as well (Partridge *et al*., 2000). It is possible that Bir1p depletion results in perturbation of the outer kinetochore structure, thereby disrupting the function of heterochromatin proteins, which may thus lead to altered kinetochore behavior in anaphase. In future, it will prove interesting to investigate the status of heterochromatin regions in cells devoid of functional Bir1p.

Alternatively, the presence of lagging chromosomes in *bir1* mutants may arise due to the absence of Ark1p on kinetochores. Recent evidence in *S. cerevisiae* suggests that the Aurora B kinase is important for establishment of chromosome bi-orientation in metaphase by destabilizing kinetochore - MT interactions (Tanaka *et al*., 2002). It is possible that the absence of Ark1p on kinetochores in *bir1* mutants might impair the formation of bi-oriented chromosomes. In such a scenario, lagging may

occur due to the stable capture of individual sister-kinetochores by MTs emanating from both spindle poles, which in turn may prevent poleward movement of chromosomes.

In fission yeast, it has been shown that the directed movement of chromosomes towards spindle poles in anaphase A is regulated by the Kin I family of kinesin motors, Klp5p and Klp6p (West *et al*., 2001; West *et al*., 2002). It has been proposed that these proteins function as 'exotubulases' that foster disassembly of kinetochore MTs (West *et al*., 2001). It can be postulated that anaphase movement defects in *bir1* mutants may be a consequence of impaired function of these plus-end directed kinesins. In view of this hypothesis, it will be interesting to investigate the status of localization of these motor proteins in *bir1* mutants.

Thus, a combination of factors may affect chromosome behavior in *bir1* mutants. Future work should address the role of Bir1p in regulating these factors, thereby influencing the movement of chromosomes during anaphase.

3.7.4. Spindle elongation

This study has shown that Bir1p function is required for complete elongation of the anaphase spindle. Bir1p-lacking cells are capable of assembling and initiating elongation of the mitotic spindle. However, spindle breakdown seems to occur prior

to ‘end-to-end’ elongation. It has been previously reported that in cells with lagging chromosomes, the rate of spindle elongation is reduced to approximately half the wild-type elongation rates (Pidoux *et al*., 2000). It is possible that lagging chromosomes in Bir1p-depleted cells cause a slow down of anaphase spindle elongation. In fact, the study by Morishita *et al* (2001) shows a decrease in spindle elongation rates in the *cut17*-275 *ts* mutant as determined by time-lapse analyses. Perhaps, accumulation of all kinetochores at the SPBs is required to accelerate spindle elongation at the end of anaphase A. In the absence of Bir1p function, when anaphase B initiates prior to completion of anaphase A, spindle elongation may not proceed effectively. This phenomenon might help coordinate onset of anaphase B with the completion of anaphase A.

Alternatively, spindle elongation may simply be slowed down due to steric ‘drag’ exerted by the lagging chromosomes. However, this possibility seems unlikely in view of Condensin mutants, which display complete spindle elongation in spite of the presence of trailing chromosome masses on the anaphase spindle (Saka *et al*., 1994; Sutani *et al*., 1999).

Bir1p relocates from kinetochores to the spindle mid-zone at anaphase A to anaphase B transition (described in detail in chapter 5). In view of its localization pattern, it will be interesting to explore the possibility that the presence of Bir1p at the

mid-zone is necessary to physically stabilize the elongating anaphase B spindle. The existence of microtubule-bundling proteins at the mid-zone, such as the BimC kinesin family of proteins and PRC1 has previously been shown (Mollinari *et al*., 2002; reviewed in Sharp *et al*., 2000). These proteins are thought to contribute to force generation during elongation by stabilizing the bipolar spindle. Future work should address the role of Bir1p in anaphase spindle elongation, possibly via its regulation of bundling proteins. As the kinetochore-based functions of the chromosome passenger complex are beginning to get unraveled, it will be interesting to study the role of this complex at its other mitotic location, the spindle.

CHAPTER 4 - The N-degron approach to create temperature-sensitive mutants in *Schizosaccharomyces pombe*

This study has so far dealt with analyzing the mitotic role of the *S. pombe* Survivin homolog, Bir1p. It will prove useful to obtain good conditional mutants of *bir1*$^{+}$, so that the multiple functions of Bir1p can be studied in detail by specifically inactivating this protein prior to different stages in mitosis. In an attempt to obtain such an allele of *bir1*, a method, based on a previously described N-degron approach to create temperature-sensitive (*ts*) mutants in *S. cerevisiae* (Dohmen *et al*., 1994) was developed in *S. pombe*. This chapter will focus on the methodology of this approach and will discuss the feasibility and possible applications of this method to create conditional mutants of essential genes in *S. pombe*.

4.1. Introduction

Previously, a strategy for generating *ts* mutant alleles based on a portable, heat-inducible degradation signal, the N-degron, was described in *S. cerevisiae* (Dohmen *et al*., 1994). This strategy involves the conditional use of the N-end rule-mediated destruction machinery to target a protein of interest in the cell. The N-end rule defines the *in vivo* stability of a protein based on its N-terminal amino-acid residue (Bachmair

et al., 1986; Varshavsky, 1996). The N-end rule machinery, which is a part of the ubiquitin (Ub) system in eukaryotes (Varshavsky, 1997; Hershko and Ciechanover, 1998), targets proteins that carry N-terminal degradation signals (N-degrons). An active N-degron is a combination of a primary 'destabilizing' N-terminal amino-acid residue of a protein and an internal lysine(s) residue. The primary destabilizing residue 'attracts' the destruction machinery by serving as a binding site for the E3 ubiquitin ligase (N-recognin). This is followed by the formation of a substrate-linked multi-ubiquitin chain at the internal lysine residue. The ubiquitinated protein is subsequently targeted for degradation by the 26S proteasome (Baumeister *et al*., 1998; refer to Fig. 4.1 for scheme).

In the heat inducible degron method tested in *S. cerevisiae*, the authors exploited the property of an Ub-fusion protein to engineer an N-degron in the marker protein, Ura3p, which is an orotidine-5'-phosphate decarboxylase (Dohmen *et al*., 1994). In eukaryotic cells, it was previously found that linear Ub-protein fusions lead to rapid cleavage of Ub at the Ub-protein junction (Bachmair *et al*., 1986). This property can hence be used to generate proteins that carry an N-terminal amino-acid residue of choice. A fusion protein in which Ura3p was fused at its amino terminus to a *ts* variant of the mouse dihydrofolate reductase ($DHFR^{ts}$) was engineered with arginine as its N-terminal residue. At 37°C, a proline to leucine missense mutation at

position 66 in the DHFR^{ts} moiety caused conformational changes that exposed internal lysine residues in DHFR. This in combination with the N-terminal arginine, a primary destabilizing amino-acid residue (Varshavsky, 1996) resulted in activation of the N-degron, leading to rapid degradation of the fusion protein by the 26S proteasome machinery. Expression of the Ub-Arg-DHFR^{ts}-Ura3p fusion protein in a strain deleted for the E3 ubiquitin ligase, N-recognin, *UBR1* (Bartel *et al*., 1990), failed to confer uracil auxotrophy to cells at 37°C, establishing that degradation of the fusion protein was indeed mediated by the N-end rule pathway. A number of proteins in *S. cerevisiae* have been shown to be unstable at higher temperatures upon fusion to the Ub-Arg-DHFR^{ts} module at the N-terminus, thus demonstrating the portability of this module (Dohmen *et al*., 1994; Labib *et al*., 2000; Tercero *et al*., 2000). The heat-inducible degron method is thus a powerful system to generate a new class of temperature-sensitive mutants, known as *td* (*t*emperature *d*egron) mutants, in a gene of interest.

This study describes the construction of a temperature degron (*td*) version of the *S. pombe* protein Bir1p and demonstrates the requirement of the N-end rule machinery to effect conditional expression of Td-Bir1p. The applicability of this approach as a useful means to create temperature-sensitive mutants in *S. pombe* is also discussed.

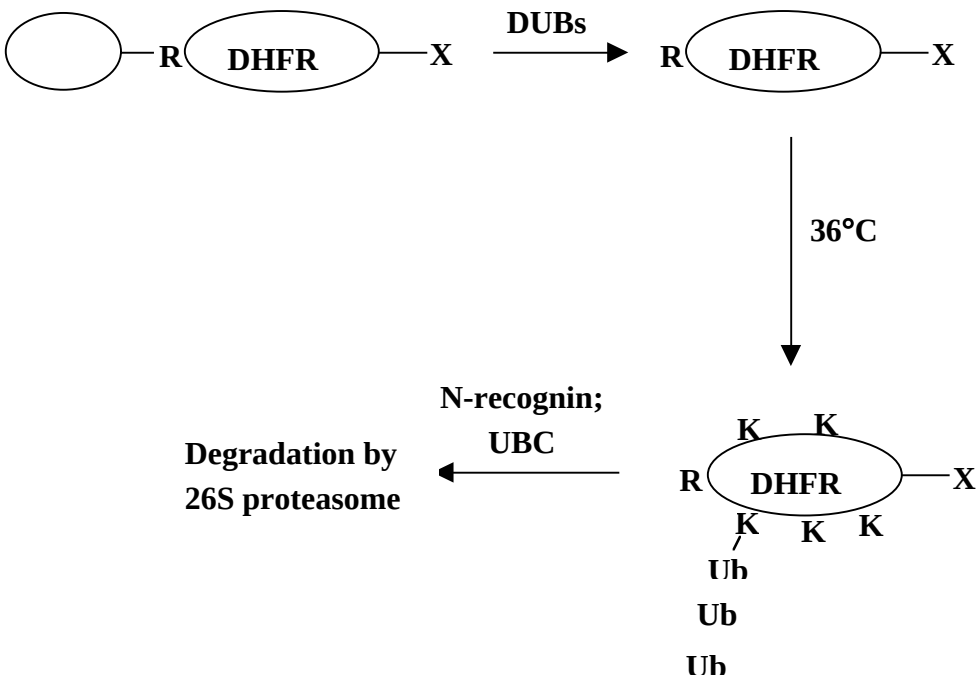

R
DHFR
X
DUBs
R
DHFR
X
36°C
N-recognin;
UBC
Degradation by
26S proteasome
K
K
R
DHFR
X
K
K
K
Ub
Ub
Ub

Figure. 4.1. The temperature-inducible N-degron method. The protein of interest “X” is fused at its N-terminus to a 76 aa ubiquitin (Ub) moiety, a destabilizing amino-acid residue, arginine (R), and a temperature-sensitive (*ts*) variant of the mouse dihydrofolate reductase (DHFR). The linear fusion to ubiquitin leads to rapid cleavage at the Ub-polypeptide junction of the fusion protein by de-ubiquitylating enzymes (DUBs). This leads to exposure of the destabilizing arginine residue at the N-terminus of the fusion pretein. The N-degron, however, remains cryptic until shift to 36°C. This shift results in unfolding of DHFR leading to exposure of its internal lysine (K) which serve as polyubiquitination sites. The arginine residue along with the exposed lysine residues (the active N-degron) attract N-end rule specific E3 ubquitin ligase (N-recognin) and ubiquitin conjugating enzymes (UBCs) which mediate targeting of the fusion protein for destruction by the 26S proteasome.

4.2. Construction of the *bir1-td* strain

The Ub-Arg-DHFR^{ts} moiety was isolated from the *S. cerevisiae* plasmid pPW66R (Dohmen *et al*., 1994), by polymerase chain reaction (PCR). This module was amplified as a 800 basepair (bp) *Xho*I-*Bgl*II fragment using the forward and reverse primers, MOH 969 and MOH 970 respectively. *gfp* was amplified as a 700bp *BamH*I-*Not*I fragment using primers MOH422 and MOH423. The first 500 nucleotides of *bir1*$^{+}$ were amplified as a *Not*I-*Sac*I fragment using primers MOH 403 and MOH 635. These three individual PCR products were assembled into the *S. pombe* integration vector, pJK210 (Keeney and Boeke, 1994), such that Ub-Arg-DHFR^{ts} module was fused at the N-terminus of GFP-Bir1p. The plasmid (pCDL675) expressing this fusion protein was linearized using *Pst*I that cut within the *bir1*$^{+}$ fragment and lithium-acetate mediated transformation (Keeney and Boeke, 1994) was used for integration at the chromosomal locus of *bir1*$^{+}$. Transformants were isolated at 24°C based on uracil prototrophy. They were then replica-plated to uracil-deficient plates containing phloxin and incubated at 36°C for 16 hours. Colonies were picked based on lethality at 36°C.

The Ub-Arg-DHFR^{ts}-GFP-*bir1*$^{+}$ strain (denoted as *bir1-td*), which grew normally at 24°C, failed to form colonies at 36°C. The *bir1-td* strain (MBY1352) was back-crossed to an *S. pombe* wild-type strain, MBY192 and a 2:2 segregation of the

ura4$^+$ marker confirmed integration of the plasmid into the genome. Integration of the N-degron tag at the *bir1*$^+$ chromosomal locus was confirmed by PCR. Live fluorescence microscopy of *bir1-td* strain at 24°C showed the localization of GFP-Bir1p to kinetochores and the spindle mid-zone in mitotic cells, consistent with the localization pattern of Bir1p in wild-type cells (data not shown). This suggested that the N-degron moiety was integrated in frame with *gfp-bir1*$^+$ at the *bir1*$^+$ chromosomal locus.

4.3. Analysis of the *bir1-td* phenotype

In order to analyze the phenotype exhibited by the *bir1-td* strain, exponentially growing cells at 24ºC were shifted to 36ºC. Cells were harvested after 4 hours, fixed and stained with DAPI-aniline blue to visualize chromosomes and the division septum. The stainings revealed that *bir1-td* cells at 24ºC exhibited wild-type morphology, growth and division pattern (Fig. 4.2). However, at 36ºC, the presence of 'stretched', mis-segregated chromosomes that were cleaved by the division septum leading to a '*cut*' phenotype, was observed (Fig. 4.2). This phenotype was identical to what has previously been described for cells depleted of Bir1p (refer to chapter 3). Immuno-fluorescence microscopy using anti-tubulin antibodies revealed incomplete anaphase B spindle elongation in *bir1-td* cells at 36ºC, also consistent with the

previous analysis of *bir1* mutants (Fig. 4.3B). These results suggested that the *bir1-td* strain behaved as a *bona fide* temperature-sensitive mutant of *bir1*$^{+}$.

4.4. Chromosome segregation defects in *bir1-td* cells is a consequence of Bir1p degradation

It was checked whether the phenotype exhibited by the *bir1-td* strain at 36ºC was due to a lack of Bir1p protein in the cell, assuming that the N-end rule mediated destruction machinery was activated. For this purpose, exponentially growing *bir1-td* cells at 24ºC were shifted to 36ºC for 3.5 hours, fixed and stained with a-GFP and a-tubulin antibodies. A culture of *bir1-td* cells at 24ºC was simultaneously fixed and stained as control. *bir1-td* cells at 24ºC displayed GFP-Bir1p localization to kinetochores in metaphase and to the spindle mid-zone in anaphase B (Fig. 4.3A).

Interestingly, in a majority of cells shifted to the restrictive temperature of 36ºC, GFP-Bir1p fluorescence on both kinetochores and the spindle mid-zone was completely abolished in mitosis (Fig. 4.3B). This result suggested that the mitotic defects, such as chromosome segregation and spindle elongation, observed in the *bir1-td* strain could be attributed to degradation of Bir1p at 36ºC.

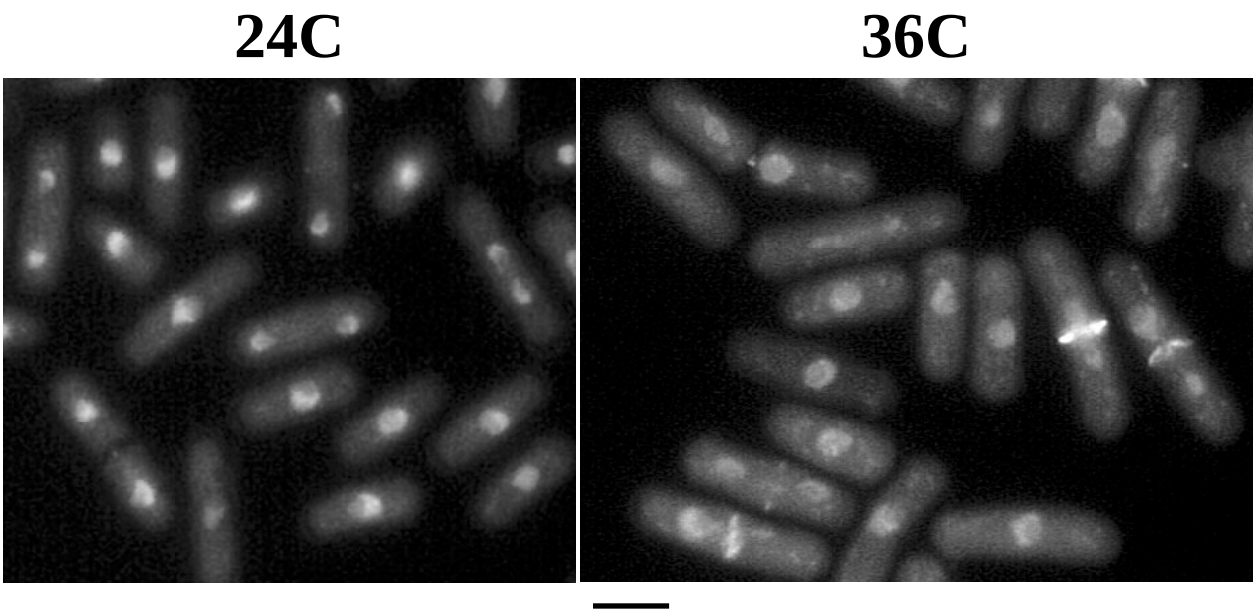

24C
36C

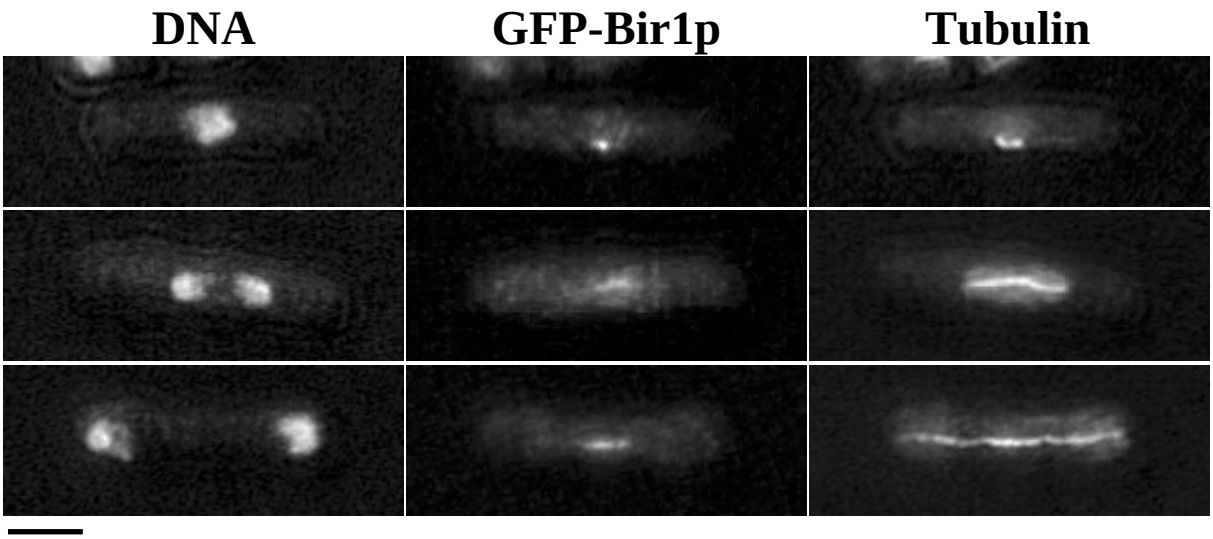
Figure. 4.2. *bir1-td* phenotype. *bir1-td* cells were to grown to exponential growth phase at 24°C and shifted to 36°C for 4 hours, fixed with formaldehyde and stained with DAPI-Aniline Blue to visualize chromosomes and cell wall/septum. Scale bar = 4.9μm.

4.5. Degradation of Bir1p in *bir1-td* cells is executed by the N-end rule mediated destruction machinery.

4.5.1. Identification and preliminary analysis of two putative N-end recognizing E3 ubiquitin ligases in *S. pombe*

It was important to establish that the loss of Bir1p observed at 36ºC in *bir1-td* cells, was indeed mediated by the N-end rule pathway. For this purpose, the status of Bir1p degradation was checked in a strain that was deleted for an essential component of the N-end rule machinery. In *S. cerevisiae*, it has previously been shown that deletion of the E3 ubiquitin ligase (N-recognin), *UBR1*, abolished N-degron-mediated degradation of a protein of interest (Dohmen *et al*., 1994). Sequence homology searches using the NCBI protein search revealed two putative N-end recognizing E3 ubiquitin ligases in *S. pombe*, SPBC19C7.02 (*ubr1*$^+$) and SPAC15A10.11 (*ubr11*$^+$). Strains deleted for *ubr1*$^+$ and *ubr11*$^+$ have been previously described (Kitamura *et al*., 2001). A PCR-based deletion strategy was used to independently create *ubr1Δ* and *ubr11Δ* strains in this study. A *ura4*$^+$ PCR product containing 80 bp of sequence homology to the flanking regions of *ubr1*$^+$ or *ubr11*$^+$ was amplified using primers BC17C702F and BC17C702R for *ubr1*$^+$ and AC15A1011F and AC15A1011R for *ubr11*$^+$. The respective *ura4*$^+$ PCR fragments were integrated at the *ubr1*$^+$ or *ubr11*$^+$

A) 24C



B) 36C

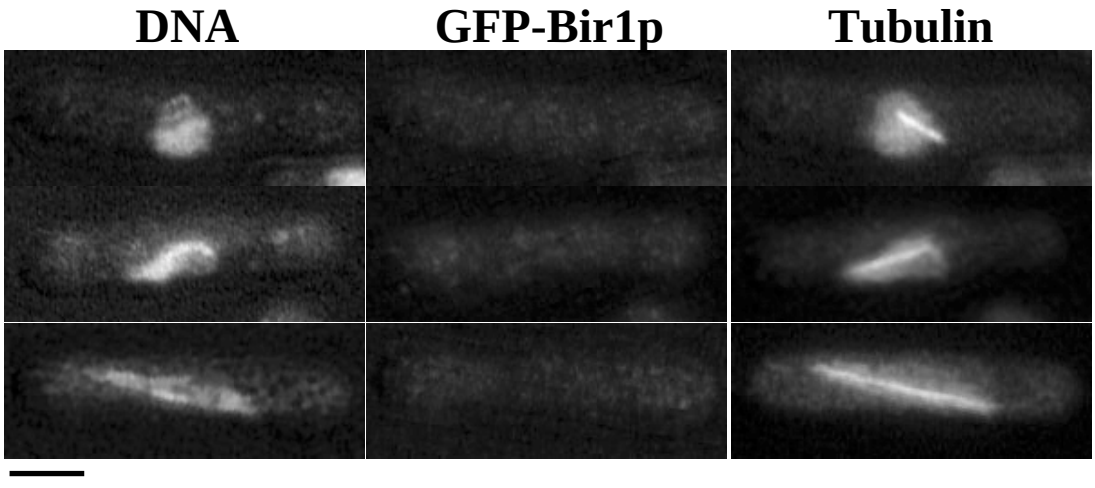


Figure. 4.3. Degradation of Bir1p-TD at 36°C. A) Exponentially growing *bir1-td* cells at 24°C were fixed with formaldehyde and stained with DAPI to visualize chromosomes, α-GFP antibodies to visualize GFP-Bir1p and TAT1 antibodies to visualize microtubules. B) Exponentially growing *bir1-td* cells were shifted to 36°C for 3.5 hours, fixed with formaldehyde and stained with DAPI to visualize chromosomes, α-GFP antibodies to visualize GFP-Bir1p and TAT1 antibodies to visualize microtubules. Scale bar = 3.7μm.

chromosomal locus of a wild-type diploid strain (MBY101/MBY104) by homologous recombination. PCR was used to confirm the complete disruption of *ubr1*$^{+}$ or *ubr11*$^{+}$ coding region by the *ura4*$^{+}$ marker gene. Sporulation of the diploid transformants revealed that both *ubr1*$^{+}$ and *ubr11*$^{+}$ were not essential for vegetative growth of *S. pombe*.

In order to check their morphology and growth pattern, *ubr1Δ* (MBY1418) and *ubr11Δ* (MBY1419) strains were grown to exponential phase in EMM medium lacking uracil. The cells were fixed and stained with DAPI and aniline-blue to visualize chromosomes and the division septum. It was apparent that *ubr1Δ* cells were delayed at some stage of the cell cycle, judging from their elongated cell morphology. However chromosome segregation and cell division appeared to occur normally (Fig. 4.4). On the other hand, *ubr11Δ* cells appeared to have wild-type cell morphology, growth and division pattern and chromosome segregation appeared normal (Fig. 4.4). Therefore, *ubr11Δ* cells were chosen for further analysis.

4.5.2. Destruction of Bir1p occurs via the N-end rule pathway

In order to test whether the N-end rule machinery mediated destruction of Bir1p in *bir1-td* cells at 36ºC, a double mutant strain of *bir1-td ubr11Δ* (MBY1486) was constructed. Interestingly this double mutant remained viable and capable of

colony formation at 36ºC. In order to analyze the status of Bir1p degradation and chromosome segregation, *bir1-td ubr11Δ* cells grown to exponential phase at 24ºC were shifted to 36ºC for 4 hours. The cells were fixed with formaldehyde and stained with α-GFP and α-tubulin antibodies. In these cells, chromosome segregation and spindle elongation occurred normally. GFP-Bir1p localized to the kinetochores and the spindle mid-zone in mitotic cells indicating that Bir1p was not degraded in these cells at 36ºC (Fig. 4.5A). A comparison of the proportion of cells displaying a '*cut*' phenotype between *bir1-td* and *bir1-td ubr11*Δ cells upon shift to 36ºC (Fig. 4.5B), clearly demonstrated the rescue of the *bir1*-related mitotic defects in the *bir1-td ubr11*Δ strain. These results effectively establish that the N-end rule pathway mediates degradation of Bir1p at 36ºC in the *bir1-td* strain. Deletion of an essential component of the N-end rule pathway abolishes Bir1p degradation possibly because of a failure to recognize the N-degron signal on Bir1p.

4.6. Discussion

This study has demonstrated that the N-degron mediated approach for construction of *ts* mutants is feasible in *S. pombe*. This method was tested using the *S. pombe* Survivin homolog, Bir1p. It was shown that N-terminal addition of an Ub-Arg-DHFR^{ts} moiety to Bir1p rendered temperature-sensitivity to cells.

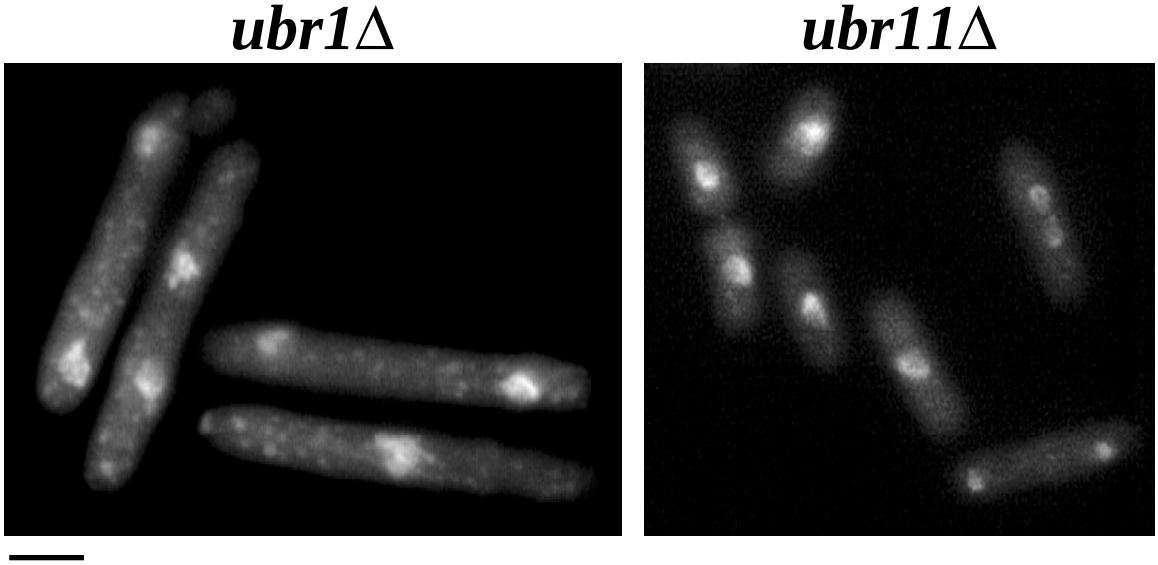

ubr1Δ
ubr11Δ

Figure. 4.4. N-end rule related E3 ubiquitin ligases (N-recognin) in *S. pombe*. Haploid deletion strains of *ubr1* and *ubr11*, the two N-recognins in *S. pombe* identified by sequence homology searches, were grown to exponential growth phase at 32°C, fixed with formaldehyde and stained with DAPI to visualize chromosomes. Scale bar = 5.8μm.

This moiety apparently serves as an N-degron signal at higher temperatures, to activate the proteasome machinery to cause Bir1p degradation.

4.6.1. The N-end rule pathway in *S. pombe*

The N-end rule pathway has previously been shown to operate in *E. coli* (Tobias *et al*., 1991), in *S. cerevisiae* (Bachmair and Varshavsky, 1989) and in mammals (Gonda *et al.*, 1989; Kwon *et al*., 2001). This study has demonstrated that a functional N-end rule pathway exists in *S. pombe*. The N-end rule pathway is currently known to operate in the nucleus and the cytosol (Varshavsky, 1996). With the use of the nuclear protein, Bir1p, this study clearly indicates that this pathway is functional in the nucleus in *S. pombe*. Recently, another *S. pombe* nuclear protein, Mcm4p, has been shown to be targeted for destruction by the N-end rule pathway when attached to an N-degron (Lindner *et al.*, 2002). It is likely that cytosolic proteins in *S. pombe* are also susceptible to N-degron mediated destruction, although this has not been tested in the present study.

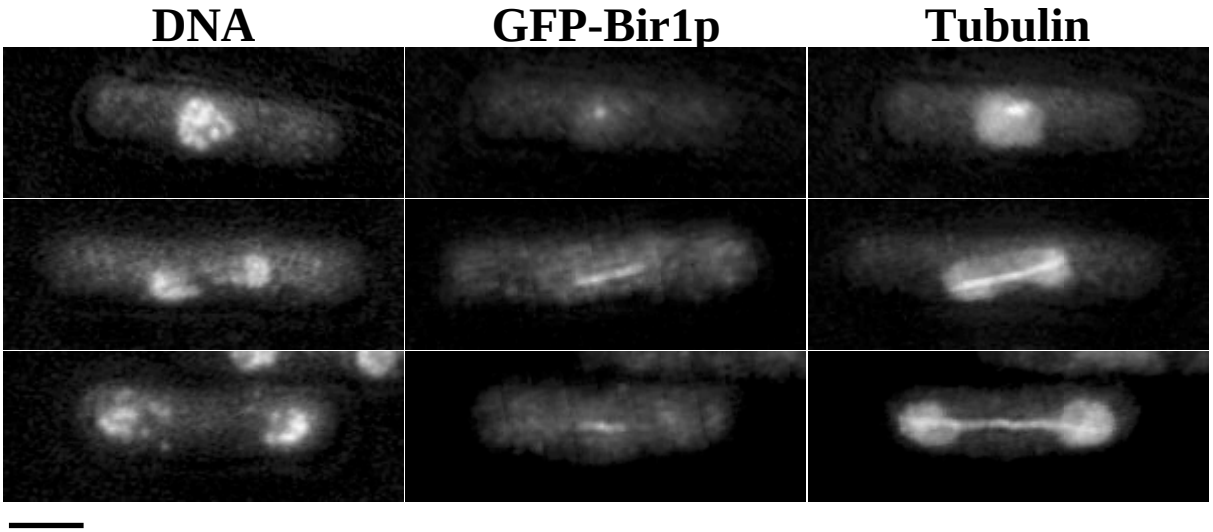
The E3 ubiquitin ligase that recognizes the N-degron signal on a protein and targets it to destruction is termed the N-recognin. *S. cerevisiae* contains one N-recognin, Ubr1p (Bartel *et al*., 1990). In *S. pombe*, two putative N-recognins have been identified based on sequence homology searches, Ubr1p and Ubr11p. The role

of Ubr11p in relieving N-degron mediated regulation of a protein has been demonstrated in this study. It is presently unclear whether Ubr1p plays a role in N-end mediated destruction, although it appears that it may be involved in some aspect of cell cycle regulation. Future studies should address this issue.

4.6.2. Applications of the N-degron mediated approach in *S. pombe*

The use of conditional mutant alleles has been a commonly used approach to address the function of any gene of interest. Several techniques have been used to generate conditional mutants in *S. pombe* of which temperature-sensitive (*ts*) mutants have by far been the most frequently used to study gene function. With the advent of reverse genetics, generating conditional mutant alleles in a gene of interest is a prime requirement to establish function of essential genes. However, the fact that not all genes are equally amenable to the generation of a conditional mutant gene product places severe limitations to this approach. There also exists the problem of mutations being "leaky" at the permissive temperature. Alternative means of conditional gene expression in *S. pombe* has been achieved with the use of thiamine-regulated *nmt1* promoters (Basi *et al*., 1993). However, thiamine-dependent 'shut-off' of gene expression is usually achieved only after passage of cells through a few cell cycles.

A)


DNA
GFP-Bir1p
Tubulin

B)

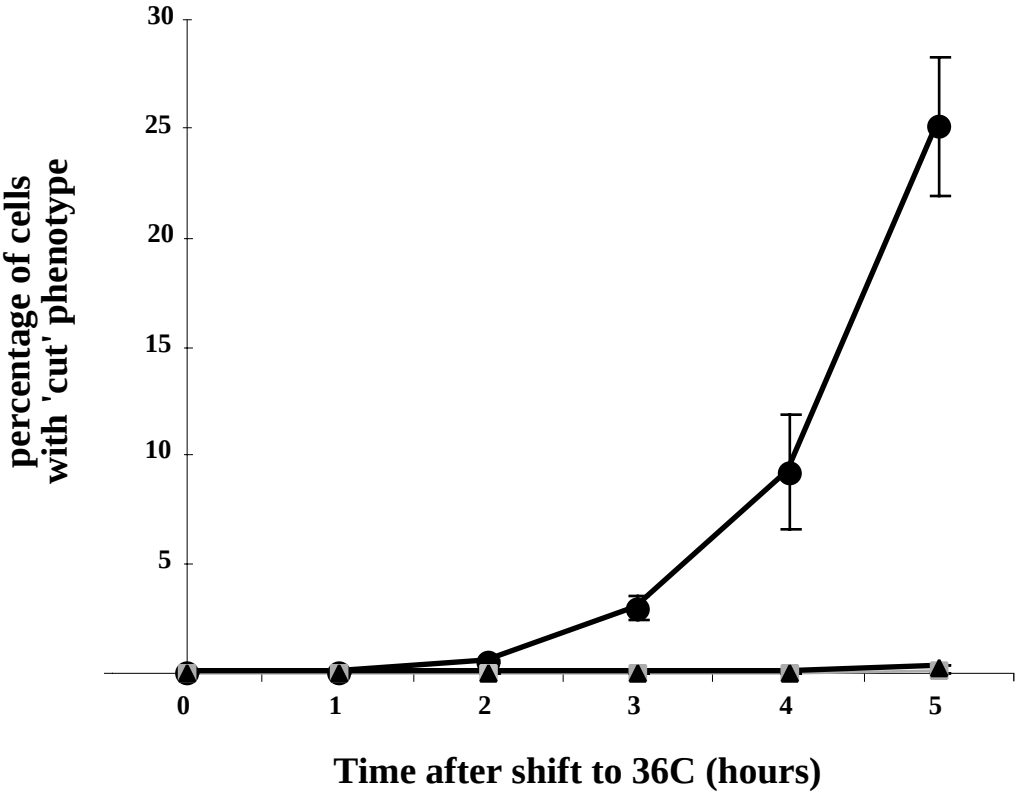

percentage of cells with 'cut' phenotype
30
25
20
15
10
5
0
1
2
3
4
5
Time after shift to 36C (hours)

Figure. 4.5. Degradation of Bir1p-TD is mediated by the N-end rule pathway. A) Degradation of GFP-Bir1p did not occur in a *bir1-td, ubr11Δ* strain. *bir1-td, ubr11Δ* cells were grown to exponential phase at 24°C and shifted to 36°C for 4 hours. The cells were fixed with formaldehyde and stained with DAPI to visualize chromosomes, α-GFP antibodies to visualize GFP-Bir1p and TAT1 antibodies to visualize microtubules. B) Chromosome segregation defects were not observed in a *bir1-td, ubr11Δ* strain grown at 36°C. *bir1-td* (●); *bir1-td, ubr11Δ* (▲); and *ubr11Δ* (■) cells were grown to exponential phase at 24°C and shifted to 36°C. Samples were taken at 0, 1, 2, 3, 4 and 5 hours after shift-up to 36°C, fixed and stained with DAPI-Aniline Blue to visualize chromosomes and cell-wall/septum. Samples of all three strains from each time-point were counted for the proportion of cells displaying a 'cut' phenotype. n=300 cells counted for each time-point. Scale bar = 3.6μm.

This study has demonstrated that conditional degradation of a protein of interest can be achieved by the use of the N-end rule pathway in *S. pombe*. It has been shown that this pathway functions in the nucleus in *S. pombe*. It will be interesting to test whether this pathway functions in the cytosol as well. The N-degron method thus makes it possible to obtain *ts* mutants for a wide variety of essential genes in S. pombe for which conditionally lethal mutants are unavailable.

Another useful advantage of the N-degron method in *S. pombe* is the availability of an internal control in the form of the *ubr11*Δ strain, which is impaired for a functional N-end rule pathway. Expression of the *td* version of a protein of interest in strains with or without a functional N-end rule pathway would allow a good comparison of the loss-of-function phenotype at restrictive conditions.

4.6.3. Variants of the N-degron method

One of the improved variants of the N-degron method previously reported in *S. cerevisiae* was developed in an attempt to overcome problems of inefficient degradation by the N-end rule pathway that were encountered in the case of some proteins (Labib *et al.*, 2000). This variant approach involved expression of the *td* version of the mini-chromosome maintenance protein, Mcm7p, in a strain over-expressing Ubr1p under the control of a galactose-inducible promoter, GAL1-10.

Mcm7p-TD was rapidly degraded at 36°C in cells induced for high levels of GAL-UBR1 expression. An attempt was made at employing a similar strategy in this study as well. However, over-expression of Ubr11p in a bir1-td strain did not significantly enhance the bir1 phenotype (data not shown). In fact, prolonged over-expression of Ubr11p by itself resulted in failed cytokinesis in a small proportion of cells (data not shown). Hence, this approach to enhance N-degron mediated destruction of proteins of interest was not pursued any further.

Another variation of the N-degron method has been recently described in *S. pombe* in the analysis of a mini-chromosome maintenance protein Mcm4p (Lindner *et al*., 2002). In this case, a degron signal was fused to a temperature-sensitive mutant allele of the *mcm4*$^{+}$ gene. The gene product of *mcm4-td* was more efficiently degraded than that of *mcm4*$^{+}$*-td* and the *mcm4-td* mutant showed a much stronger phenotype than the *ts mcm4* mutant at 36°C. Such variant approaches offer much scope for the utilization of the N-degron method for obtaining conditional degradation of a variety of proteins in *S. pombe*.

4.6.4. Limitations of the N-degron approach

One of the main limitations of this method is the restriction of its applicability to only those proteins that can tolerate an N-terminal tag. Also, at present only nuclear

proteins have been shown to be degraded by the N-end rule pathway and it is unclear if proteins that reside in organelles and other membranes can be targeted for degradation by this approach. Variability in degradation rates depending on the inherent stability of the protein of interest could also pose problems.

In spite of these limitations, the N-degron mediated approach could prove to be a powerful tool to achieve conditional gene expression in *S. pombe*.

CHAPTER 5 - An analysis of the cellular localization pattern of Bir1p

The chromosome-passenger complex of proteins, in higher eukaryotes, exhibits a dramatic pattern of localization during mitosis. Located at kinetochores at the onset of mitosis, this complex relocates to the spindle mid-zone in anaphase and eventually to the mid-body and cleavage furrow during cytokinesis (Carmena and Earnshaw, 2003). It is hypothesized that this characteristic localization pattern dictates the functions of the passenger complex in coordinating nuclear and cell divisions. This chapter will focus on a detailed analysis of the cellular localization pattern of one of the members of the passenger complex, the *S. pombe* homolog of Survivin, Bir1p, in order to obtain further insight into its functions.

5.1. Bir1p, a nuclear protein, localizes to kinetochores and the spindle mid-zone during mitosis.

In order to analyze the intracellular localization of Bir1p, a strain (MBY653) was created in which the coding region of Bir1p was fused downstream of the reporter molecule, green fluorescent protein (GFP). This functional fusion protein, the sole

copy of Bir1p in the cell, was under the control of native *bir1+* regulatory elements so that wild-type levels of GFP-Bir1p expression could be achieved.

An asynchronous population of cells expressing GFP-Bir1p was grown at 32ºC and the localization of Bir1p was observed. GFP-Bir1p was detected at several distinct cellular locations in a cell-cycle dependent manner. Consistent with a previous study (Uren *et al*., 1999), Bir1p was localized to the nucleus at all stages of the cell cycle. Additionally, GFP-Bir1p was prominently detected in 'spot' like structures in the vicinity of chromosomes and also localized to a structure resembling the mitotic spindle (Fig. 5.1A). Microtubule and nuclear staining of these cells established that in early mitotic cells that contained a short spindle, GFP-Bir1p brightly stained one to three spots. Upon entry into anaphase, Bir1p was no longer detected on spots, but was found localized to the mid-zone of the elongating anaphase spindle, possibly at the region of microtubule overlap (Fig. 5.1B).

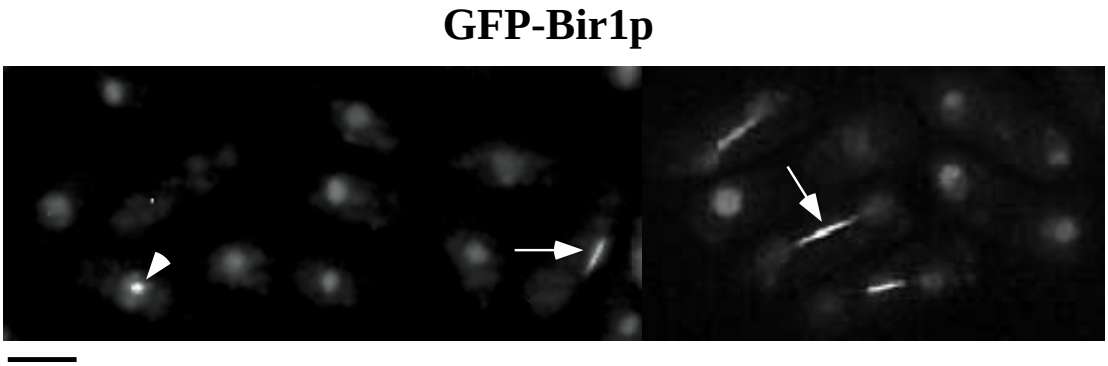
The spot localization of Bir1p was reminiscent of the pattern of localization exhibited by resident kinetochore proteins, such as Mis6p and Mis12p (Saitoh *et al*., 1997; Goshima *et al*., 1999). In order to test whether Bir1p indeed localized to kinetochores, GFP-Bir1p staining was analyzed in haploid (MBY686) and diploid (MBY776) β-tubulin mutant (*nda3*-KM311) cells at the restrictive temperature of 19°C. At this temperature, *nda3*-KM311 cells arrest at metaphase with condensed chromosomes and

are most suitable for visualizing kinetochores (Funabiki *et al*., 1993). GFP-Bir1p localized prominently to three spots in the haploid and to six spots in the diploid *nda3*-KM311 strain (Fig. 5.2A and B). A correlation between the number of GFP-Bir1p spots and the number of chromosomes indicated that Bir1p was indeed a component of the kinetochore. To further reaffirm this idea, co-localization studies of Bir1p with a previously described kinetochore protein, Mis6p (Saitoh *et al*., 1997) were done. For this purpose, a *nda3*-KM311 strain expressing GFP-Bir1p and Mis6p-13myc fusion proteins was constructed (MBY818) and the localization of Bir1p in relation to Mis6p was assessed upon cold arrest. Bir1p co-localized with Mis6p to a large extent (Fig. 5.2 C), thus strongly supporting the idea that Bir1p localizes to kinetochores. In summary, Bir1p is a nuclear protein that localizes to kinetochores prominently in early mitosis and moves to the mid-zone of the elongating spindle during anaphase.

5.2. Localization of Bir1p during meiotic division

Since Bir1p localized to kinetochores and the spindle mid-zone, essential structural components of the chromosome-segregation machinery, the localization of Bir1p was examined in meiotic cells that undergo both reductional (meiosis I) and equational (meiosis II) divisions to segregate chromosomes.

A



B

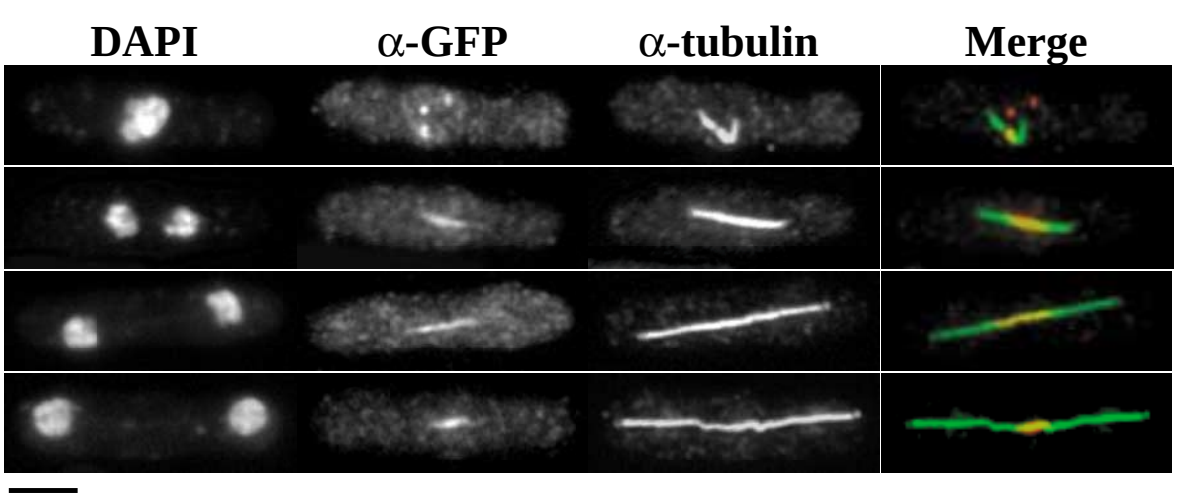


Figure. 5.1. Cellular localization of GFP-Bir1p. A) GFP-Bir1p-expressing cells were grown to exponential growth phase at 32°C in complete medium to visualize GFP epi-fluorescence. The arrowhead indicates localization of Bir1p to spots and arrows indicate localization of Bir1p to the spindle. Scale bar = 5.2μm.
B) Exponentially growing cells expressing GFP-Bir1p were fixed with formaldehyde and stained with DAPI to visualize chromosomes, α-tubulin to visualize microtubules and α-GFP to visualize GFP-Bir1p. Merge represents an overlap of GFP (red) and microtubule (green) staining. Scale bar = 3.7μm.

A homothallic strain that expressed GFP-Bir1p was constructed (MBY1222) and induced to undergo synchronous meiosis and localization of Bir1p was monitored through various stages of meiosis I and II. Growth in nitrogen-free medium for 5 to 6 hours led to the formation of conjugated cells. These cells showed the presence of nuclei that displayed an elongated morphology characteristic of the previously described 'horse-tail' nuclear structure in cells undergoing pre-meiotic DNA synthesis and meiosis I prophase (Robinow, 1977). In cells with horsetail nuclei, GFP-Bir1p localized to a spot that appeared to be at the edge of the nuclear material (Fig. 5.3 i). This staining was reminiscent of the pattern of telomere clustering at the spindle pole body (SPB) that leads the horsetail movement of the meiotic nucleus (Chikashige *et al*., 1994). It is currently unclear whether Bir1p localizes to the clustered telomeres at the SPB or whether it is associated with the SPB itself during this stage of meiosis. At the onset of metaphase in meiosis I, when sister chromatids segregate to the same spindle pole, cells showed the presence of condensed chromosomes and a short spindle similar to mitotic metaphase. At this stage, GFP-Bir1p localized to distinct spots (Fig. 5.3 ii) that were strikingly similar to the pattern of kinetochore localization in meiotic cells (Bernard *et al*., 2001a). Subsequently GFP-Bir1p relocated to the mid-zone of the elongating meiotic spindle (Fig. 5.3 iii & iv).

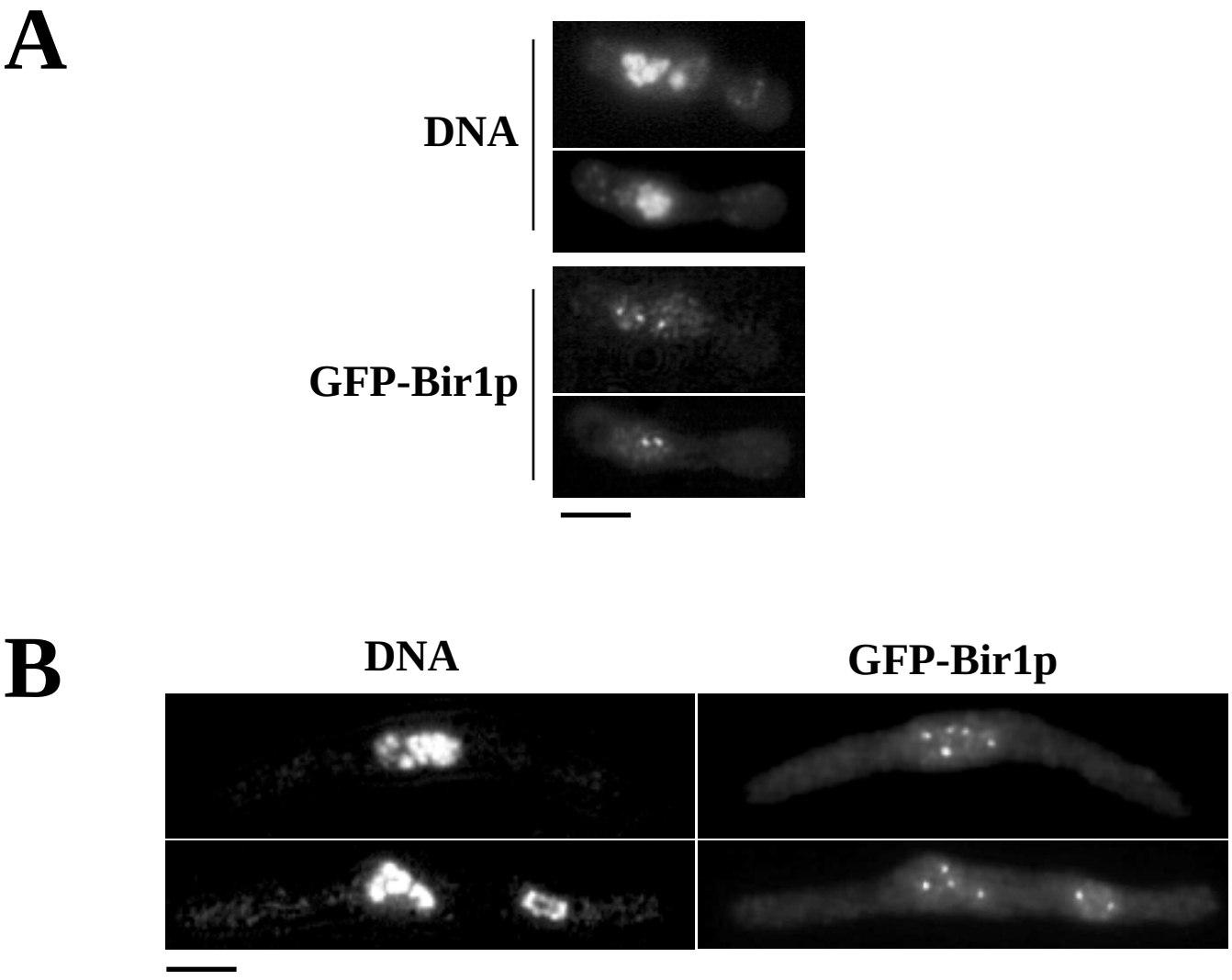

A
DNA
GFP-Bir1p
B
DNA
GFP-Bir1p
C
DNA
Bir1p
Mis6p
Merge
2 μm
2 μm

Figure. 5.2. Bir1p localizes to kinetochores in mitosis. A haploid *nda3*-KM311 strain (A) and a diploid *nda3*-KM311/*nda3*-KM311 strain (B) expressing GFP-Bir1p were grown to exponential growth phase at 32°C and shifted to 18°C for 6 hours, fixed and stained with DAPI to visualize chromosomes and α-GFP to visualize GFP-Bir1p. C) *nda3*-KM311 cells expressing GFP-Bir1p and Mis6-13Myc fusions were grown to exponential growth phase at 32°C and shifted to 18°C for 6 hours, fixed and stained with DAPI to visualize chromosomes, α-GFP to visualize GFP-Bir1p and α-myc to visualize Mis6-13Myc. (Bottom) Enlarged merged images of GFP-Bir1p (red) and Mis6-13Myc (green). Scale bar = 4.5μm.

Furthermore, during meiosis II, an identical sequence of kinetochore and mid-spindle localization of GFP-Bir1p was observed (Fig. 5.3 v-vii).

Thus, it can be concluded that Bir1p localizes in a similar fashion during mitotic and meiotic chromosome segregation. Additionally, Bir1p possibly localizes to the leading edge of the horsetail nucleus suggesting that this protein may also be located at telomeres or the SPB during meiosis.

5.3. Bir1p localizes to centromeres during interphase

In order to assess the localization of Bir1p during interphase, a strain was constructed in which GFP-Bir1p was expressed in a *cdc25*-22 mutant background (MBY683). Upon shift to 36ºC, *cdc25*-22 cells arrest at G2-M transition due to a failure in activation of the cyclin dependent kinase (CDK), Cdc2p (Russell and Nurse, 1986). In G2 arrested cells, GFP-Bir1p localized to more than one spot in the nucleus (Fig. 5.4A). To test whether this localization was specific to G2, the localization of Bir1p was analyzed in cells that were arrested in mid S-phase due to the addition of 12 mM hydroxyurea (HU). After 8 hours of HU-induced arrest at 32ºC, GFP-Bir1p was detected on more than one spot in the nucleus (Fig. 5.4B). Since centromeres cluster at the SPB during interphase and are detected as one spot (Funabiki *et al*., 1993), it was checked whether any of the Bir1p spots corresponded to clustered centromeres at

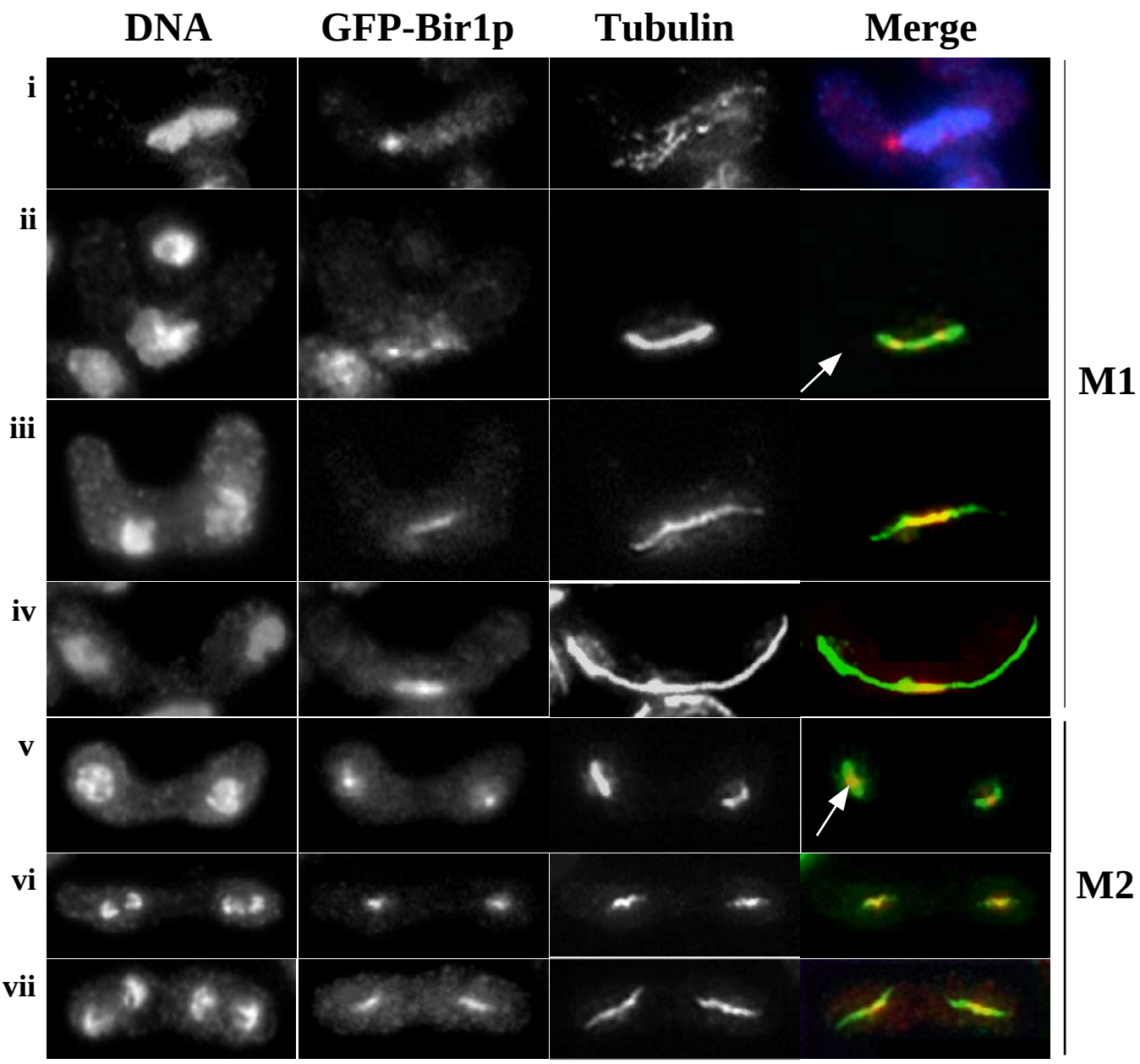

DNA
GFP-Bir1p
Tubulin
Merge
i
ii
iii
iv
v
vi
vii
M1
M2

Figure. 5.3. Localization of GFP-Bir1p to the kinetochores and the spindle mid-zone in meiosis I and II. A homothallic GFP-Bir1p expressing strain grown to stationary phase at 36°C in minimal medium lacking glucose was induced to undergo synchronous meiosis by shift to medium lacking nitrogen. Samples were taken at 6, 7, 8, 9 and 10 hours after shift to nitrogen-free medium. Cells were fixed and stained with DAPI to visualize chromosomes, α-GFP to visualize GFP-Bir1p and α-tubulin to visualize microtubules. M1 and M2 refer to meiosis I and II respectively. The panels represent cells in meiosis I prophase (i), metaphase I (ii), anaphase I (iii and iv), metaphase II (v), and anaphase II (vi and vii). Merge i represents an overlap of GFP (red) and DAPI (blue) staining. Merge ii-vii represent an overlap of GFP (red) and microtubules (green) staining. The arrows indicate spotty localization of GFP-Bir1p in metaphase I and II, possibly reflecting kinetochore staining.

the SPB. For this purpose, the MBY818 strain, that expressed GFP-Bir1p and Mis6p-13Myc, was grown at 32ºC in the presence of 12mM HU, fixed and stained with α-GFP and α-myc antibodies to localize Bir1p and Mis6p respectively. In these cells, one of the brightly stained GFP-Bir1p spots always co-localized with the Mis6p spot (Fig. 5.5). These observations suggested that Bir1p localizes to the centromeres in interphase and continues to remain localized until onset of anaphase in mitosis. The structures represented by other Bir1p spots are presently unclear.

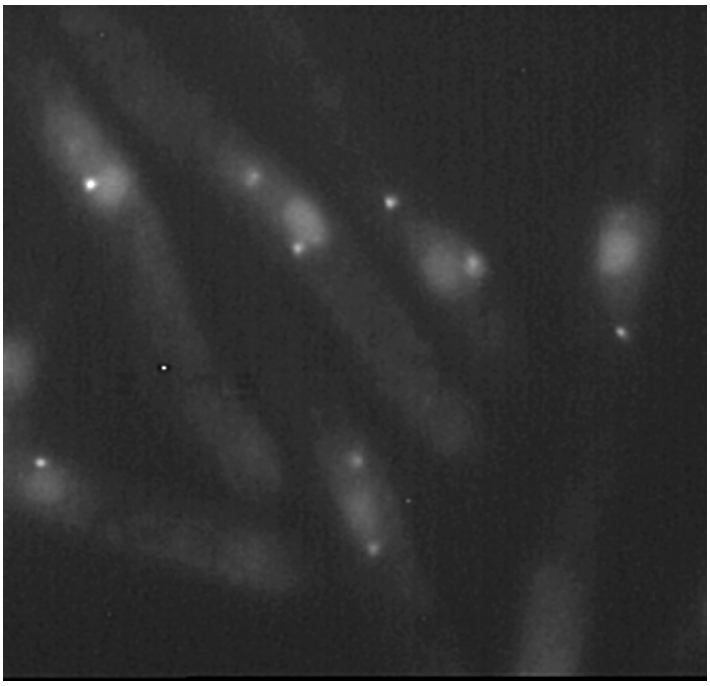
5.4. **Bir1p remains on kinetochores until completion of anaphase A and moves to the spindle mid-zone upon onset of anaphase B.**

5.4.1. Time-lapse analysis of GFP-Bir1p localization in mitotic cells

Since Bir1p was detected at two distinct locations during the course of mitosis, first at the kinetochores and then at the spindle mid-zone, the transition of Bir1p localization from one structure to the other was investigated. Wild-type (MBY653) and *cdc25*-22 (MBY683) strains expressing GFP-Bir1p were used to perform time-lapse analyses. Early mitotic cells displaying bright, multiple kinetochore spots of GFP-Bir1p were chosen to observe the localization pattern over time. Selected images for the time-lapse series in wild-type cells are shown in Fig. 5.6. GFP-Bir1p was detected as multiple spots, presumably kinetochores, along the length of the short spindle (time

A)

***gfp-bir1*$^{+}$, *cdc25*-22**



B)

***gfp-bir1*$^{+}$ HU treatment**

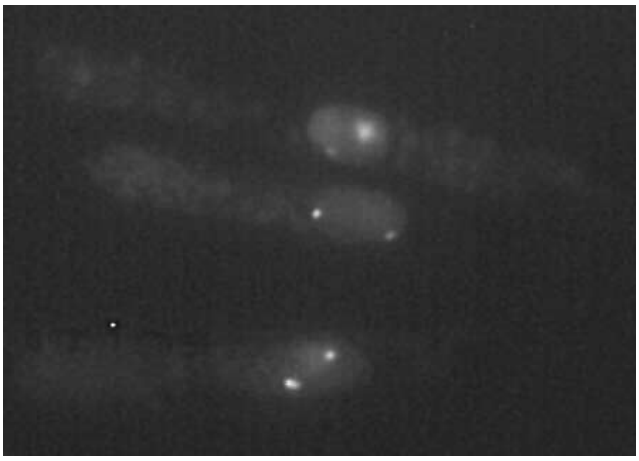


Figure. 5.4. Bir1p localizes to more than one spot during interphase. A) Exponentially growing *gfp-bir1*$^{+}$ *cdc25*-22 cells at 24°C were shifted to 36C for 4 hours. 1µl of cells were spotted on a slide and immediately visualized for GFP epi-fluorescence. B) 12mM hydroxyurea was added to exponentially growing *gfp-bir1*$^{+}$ cells for 8 hours at 32°C. 1µl of cells were spotted on a slide and immediately visualized for GFP epi-fluorescence. Scale bar = 3.1µm.

point 0s). The kinetochores then appeared to clump together at the center of the spindle (time-point 30s-arrowhead). Eventually, a striking movement of these spots occurred towards opposite ends (indicated by arrows), which was suggestive of anaphase A movement of kinetochores towards the spindle poles (time point 180-240s). Shortly thereafter, the protein was detected along the length of the spindle (time point 270-450s), and remained restricted to the mid-region of the spindle, as the nuclei migrated away from each other (data not shown).

In a more detailed analysis, *cdc25*-22 cells expressing GFP-Bir1p were synchronously released at 24ºC from G2 arrest and assessed for Bir1p localization. Images from two different time-lapse series are shown in Fig. 5.7A &B. Soon after release, prominent localization of GFP-Bir1p to a bright spot, possibly at the spindle equator, was observed (Fig. 5.7A time points 1’40’’ and 4’40’’). A few other faint GFP-Bir1p spots were also observed at this stage, the nature of which is presently unknown. Eventually, this bright spot of GFP-Bir1p split into multiple spots that moved away from each other, similar to the presumptive anaphase A movement of kinetochores earlier described in wild-type cells (Fig. 5.7A time points 5’40’’to 6’20’’). GFP staining was eventually detected on the spindle (time points 10’ and 18’). Time series 2 more clearly depicts this anaphase A movement of GFP-Bir1p-stained kinetochores towards the spindle poles (Fig. 5.7B time points 240s to 300s - arrows) before its

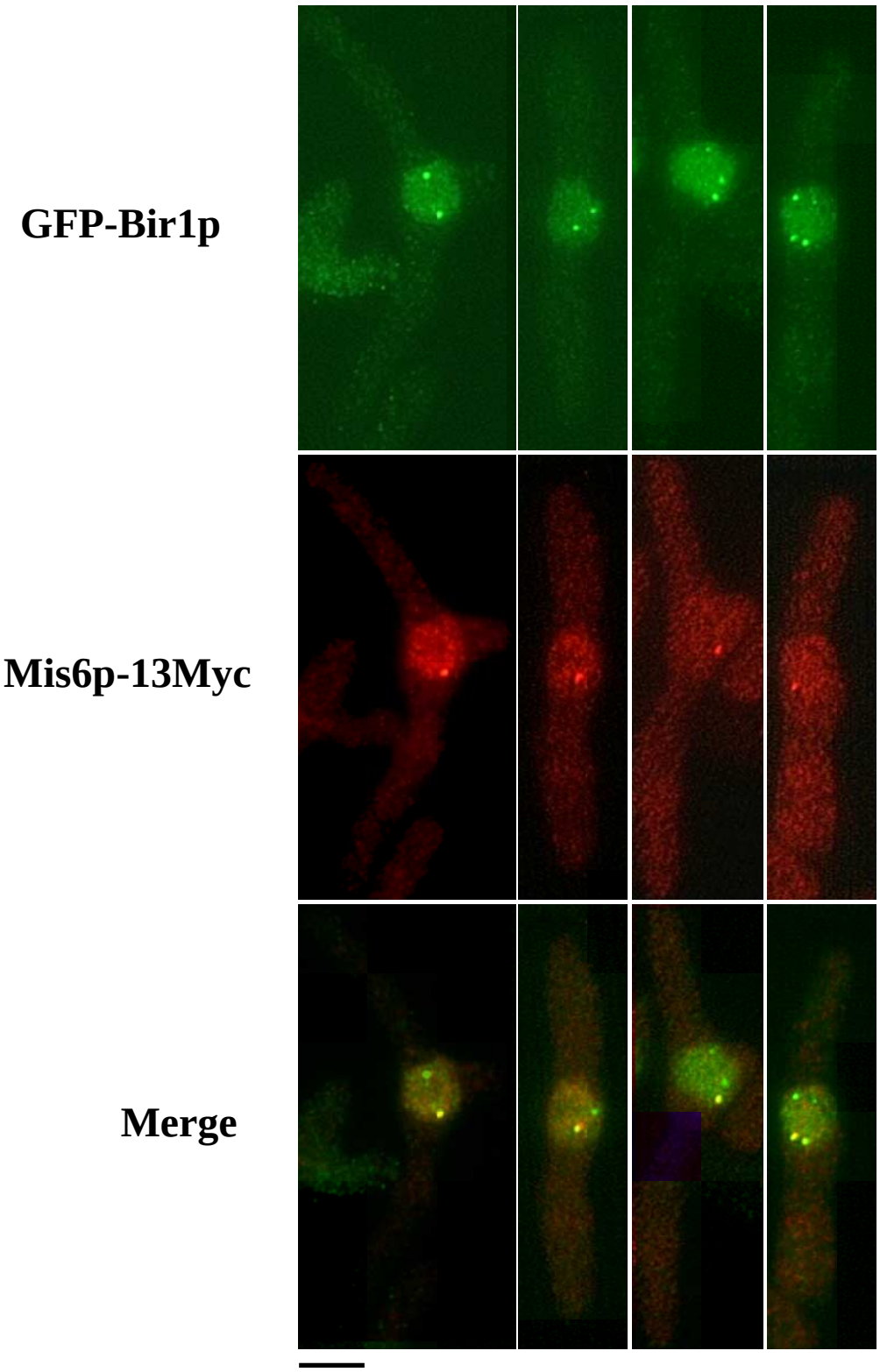

GFP-Bir1p
Mis6p-13Myc
Merge

Figure. 5.5. Bir1p colocalizes with Mis6p in interphase. 12mM hydroxyurea was added to exponentially growing *gfp-bir1*$^{+}$ *mis6*$^{+}$-13*myc nda3*-KM311 cells for 8 hours at 32°C. Cells were fixed with formaldehyde and stained with DAPI to visualize chromosomes, α-GFP to visualize GFP-Bir1p and α-myc to visualize Mis6-13Myc. Scale bar = 4.3μm.

relocalization to the spindle (Fig. 5.7B time points 390s to 480s).

Anaphase A movement of kinetochores in *S. pombe* has previously been well documented using the fluorescence in situ hybridization (FISH) technique (Funabiki *et al*., 1993). The clumping of centromeres as one bright spot, indicative of a metaphase plate-like structure and the eventual movement of sister centromeres towards opposite poles have been visualized (Funabiki *et al*., 1993). Similar dynamics exhibited by GFP-Bir1p-stained kinetochores suggested that Bir1p remains on kinetochores until completion of anaphase A and relocates to the spindle mid-zone at the onset of anaphase B.

5.4.2. Bir1p remains on kinetochores in the *klp5*Δ mutant that initiates spindle elongation prior to completion of anaphase A.

A second approach was used to investigate whether Bir1p remains on kinetochores until end of anaphase A. Two related kinesins in *S. pombe*, *klp5*$^{+}$ and *klp6*$^{+}$, though not essential for cell viability, are required for timely completion of anaphase A (West *et al*., 2002). In most *klp5*Δ and *klp6*Δ cells, lagging chromosomes are observed in early anaphase B along the length of the elongating spindle. However, the chromosomes eventually reach the poles and the cells divide with equally segregated nuclei (West *et al*., 2002). Since *klp5*Δ and *klp6*Δ cells are slowed

GFP-Bir1p

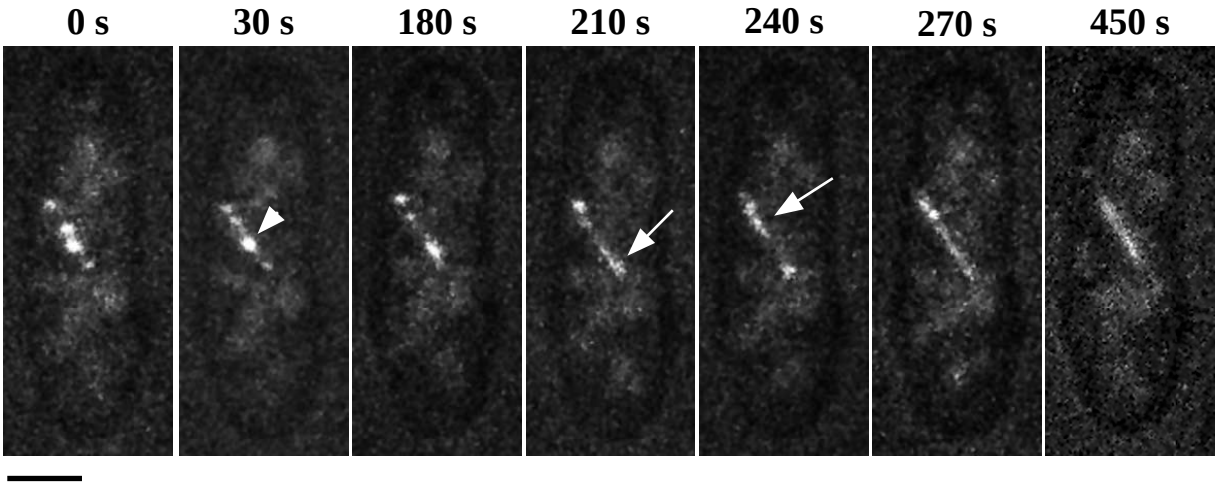


Figure. 5.6. Time-lapse analysis of wild-type *gfp-bir1*$^{+}$ cells. *gfp-bir1*$^{+}$ cells were grown to exponential growth phase and concentrated by centrifugation. 1µl of cells was spotted on a slide and immediately pressed down with a coverslip to get an even smear of cells. The edges were sealed to prevent drying. The cells were subjected to time-lapse microscopy at 24°C and images were captured at 30-second intervals. Numbers refer to time in seconds. The arrowhead indicates the bright spot of GFP-Bir1p that is suggestive of congressed metaphase kinetochores. The arrows indicate anaphase A movement of kinetochores to the spindle poles. Scale bar = 2.8µm.

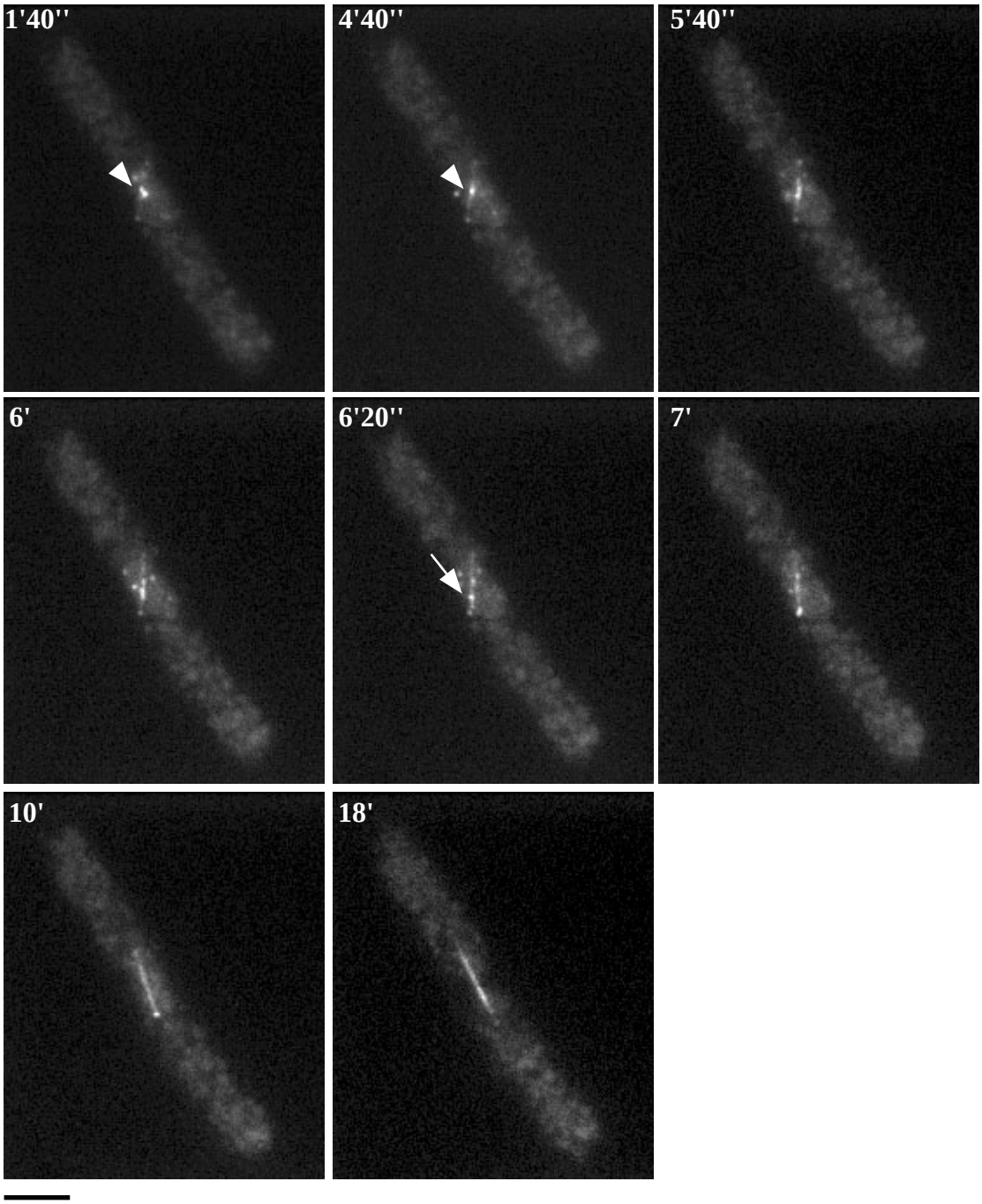
down in anaphase A, we tested the localization of Bir1p in these mutants. Exponentially growing *klp5*Δ cells expressing GFP-Bir1p (MBY1334) were fixed and stained with α-GFP and α-tubulin antibodies. In these cells, GFP-Bir1p was detected on kinetochores that co-localized with the lagging nuclear material along the length of the spindle in early anaphase B cells (Fig. 5.8). Localization of Bir1p to the spindle mid-zone was only observed in late anaphase B cells in which the chromosomes were completely segregated to the spindle poles (data not shown). Identical results were observed in *klp6*Δ cells expressing GFP-Bir1p (data not shown). Altogether, these observations suggested that Bir1p remains localized on kinetochores until the sister chromatids reach opposite spindle poles, upon which it re-distributes to the spindle mid-zone.

5.5.The kinetochore-protein pool of Bir1p moves to the spindle mid-zone in anaphase B.

Previous experiments suggested that Bir1p remains localized to kinetochores until completion of anaphase A. In this context, it was interesting to question whether the spindle mid-zone localization of Bir1p in anaphase B occurred due to redistribution of protein from the kinetochore pool, when sister chromatids reach the SPBs.

A

***gfp-bir1*$^{+}$ *cdc25*-22**



B ***gfp-bir1*$^{+}$ *cdc25*-22**

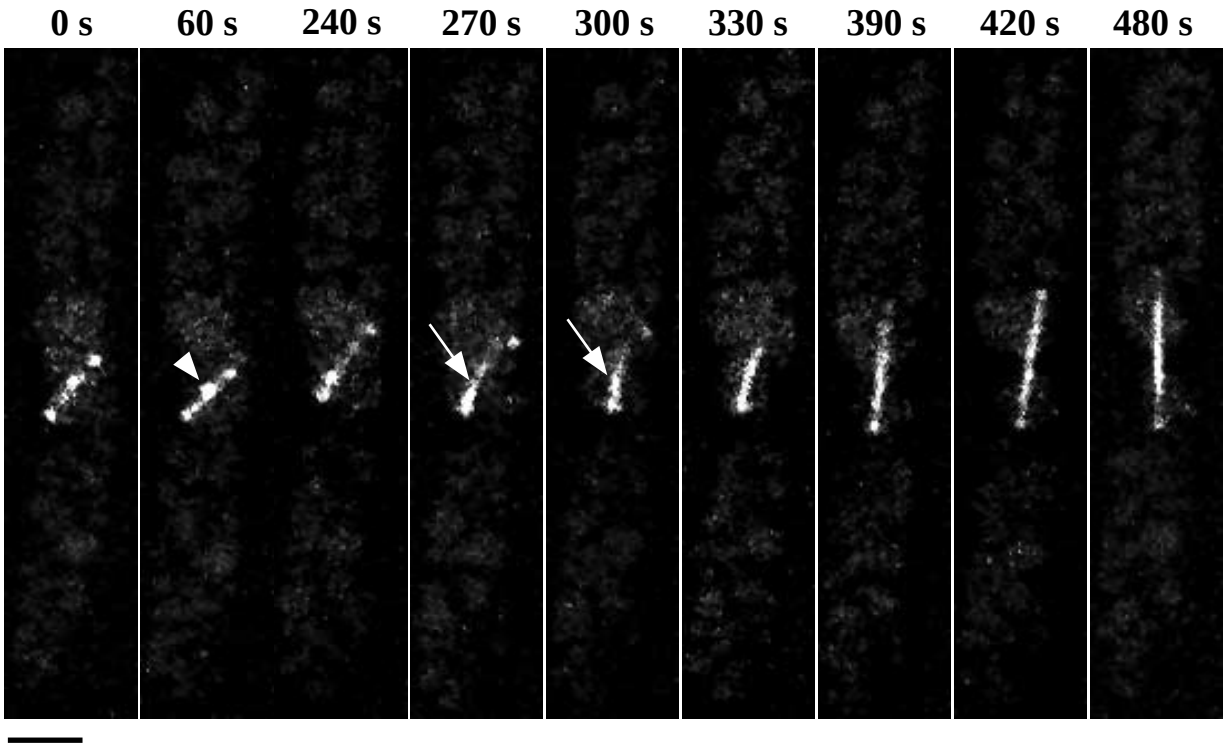


Figure. 5.7. Time-lapse analysis of *gfp-bir1*$^{+}$ *cdc25*-22 cells synchronously released into mitosis. Exponentially growing *gfp-bir1*$^{+}$ *cdc25*-22 cells at 24°C were shifted to 36°C for 4 hours. The cells were then shifted down to 24°C for 10 minutes before mounting for time-lapse analysis as described in legend of Figure. 5.6. Images were captured at 20-second (A) or 30-second (B) intervals. The arrowhead indicates the bright spot of GFP-Bir1p that is suggestive of congressed metaphase kinetochores. The arrows indicate anaphase A movement of kinetochores to the spindle poles. Scale bars = 4.3μm (A); = 2.9μm (B).

Fluorescence recovery after photo bleaching (FRAP) experiments were performed in order to address this issue. *cdc25*-22 cells expressing GFP-Bir1p (MBY683) grown at 24ºC were utilized for these experiments owing to their slightly larger size in comparison to wild-type GFP-Bir1p expressing cells. Cells displaying bright GFP spots of Bir1p, indicative of their early mitotic state, were chosen for photo bleaching (n=5 cells). A series of images from a single FRAP experiment are shown in Fig. 5.9. A region of interest confined to the area of GFP-Bir1p containing spots was subjected to 100 iterations of the 488 nm He/Ar laser at 100% power. The GFP fluorescence of Bir1p was quenched immediately after photo bleaching (time point 0'). A time-lapse analysis of the bleached cell revealed that GFP fluorescence failed to recover even after 30 minutes, although nuclear division seemed to occur normally (indicated by the nuclear outline – time points 0' to 30'). Normal passage through nuclear division indicated that the process of photo bleaching by itself did not affect cell viability. The above result on GFP-Bir1p dynamics led to two conclusions: 1) No rapid exchange of Bir1p protein occurs on kinetochores; 2) The Bir1p protein pool on the kinetochore probably redistributes to the spindle mid-zone in anaphase B.

5.6.Factors that regulate redistribution of Bir1p from kinetochores to the spindle mid-zone.

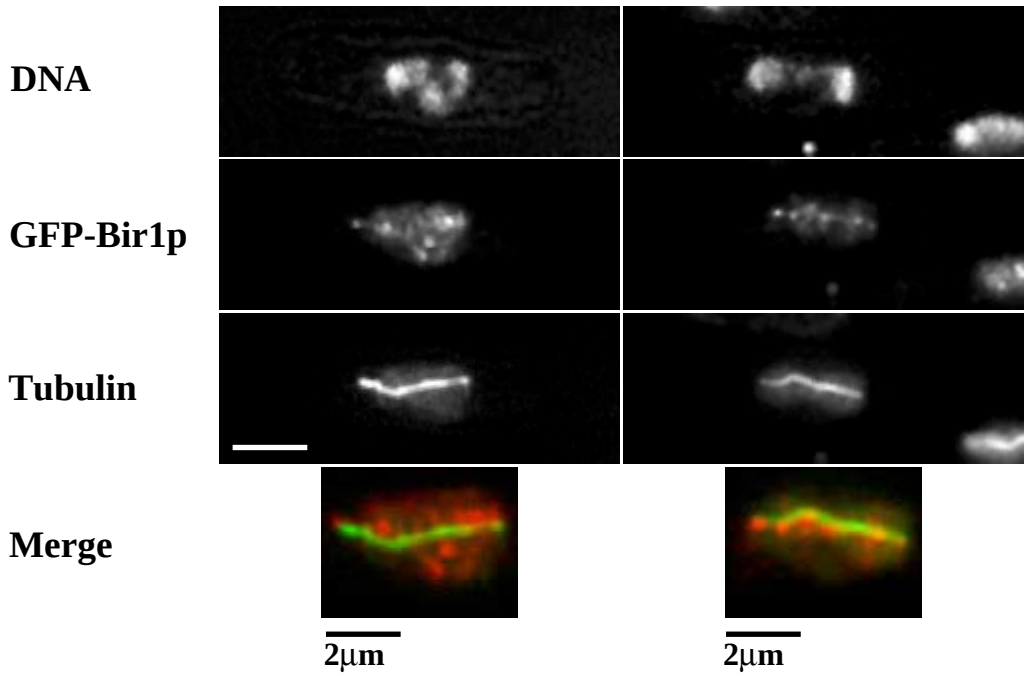

DNA
GFP-Bir1p
Tubulin
Merge
2μm
2μm

Figure. 5.8. GFP-Bir1p remains on kinetochores in *klp5*Δ mutant cells. Exponentially growing GFP-Bir1p expressing *klp5*Δ mutant cells at 32°C were fixed with formaldehyde and stained with DAPI to visualize chromosomes, α-GFP to visualize GFP-Bir1p and α-tubulin to visualize microtubules. (Bottom) Enlarged merged images of GFP-Bir1p (red) and microtubules (green). Scale bar = 3.7μm.

Time-lapse and FRAP experiments have indicated that a physical redistribution of Bir1p might be occurring from kinetochores to the spindle mid-zone in anaphase B. It can then be questioned whether this transition is regulated by certain biochemical events during mitosis, or if this was simply a structural event that occurred as a consequence of chromosomes physically reaching the spindle poles. Two of the main biochemical events that control equal segregation of chromosomes during mitosis include: 1) Loss of sister-chromatid cohesion at metaphase to anaphase transition and 2) Cyclin B destruction that allows for anaphase spindle elongation and eventual exit from mitosis. It was checked whether any of these factors played a role in Bir1p redistribution to the spindle mid-zone.

5.6.1. Lack of sister-chromatid separation may not influence Bir1p localization from kinetochores to the spindle mid-zone.

Loss of cohesion allows movement of sister-chromatids to opposite spindle poles in anaphase A (Nasmyth, 2001). Two approaches were utilized in order to test whether redistribution of Bir1p from kinetochores to the spindle was affected by the status of sister chromatid cohesion. In the first approach, a strain was constructed in which the entire coding region of the *dis1*$^{+}$ gene was deleted (MBY1427). Dis1p belongs to a highly conserved family of microtubule-associated proteins (MAPs)

known as the Dis1/TOG family (Ohkura *et al*., 2001). *dis1*$^{+}$, whose deletion confers cold-sensitivity, is important for sister-chromatid disjunction. *dis1*Δ mutants at 19ºC display hyper-condensed chromosomes that are non-disjoined (Ohkura *et al*., 1988). Mitotic spindle assembly and elongation occurs although the cells fail to exit from mitosis due to high levels of cyclin B (Ohkura *et al*., 1988; Nabeshima *et al*., 1995, Nabeshima *et al*., 1998). This mutant thus provided a seemingly suitable condition to test the dependency of spindle re-localization of Bir1p, on sister-chromatid cohesion. *dis1*Δ cells expressing GFP-Bir1p (MBY1354) were grown at 19ºC for 6 hours, fixed and stained to visualize Bir1p with respect to chromosomes and the mitotic spindle. Cells in anaphase B contained elongated spindles with condensed chromatin that was mis-segregated. In these cells, GFP-Bir1p strongly stained up to three kinetochores (indicative of non-disjunction) in spite of the presence of an elongated spindle (Fig. 5.10). This observation suggested that loss of sister-chromatid cohesion was probably important for removal of Bir1p from kinetochores. In the second approach, GFP-Bir1p localization was tested in cells that over-expressed the non-degradable version of Cut2p, Cut2Δ80p. Degradation of *S. pombe* Cut2p (securin) at the metaphase to anaphase transition is essential for sister-chromatid separation (Funabiki *et al*., 1996). Cells that mildly over-express *cut2*Δ*80* under control of the weak *nmt1*-81 promoter

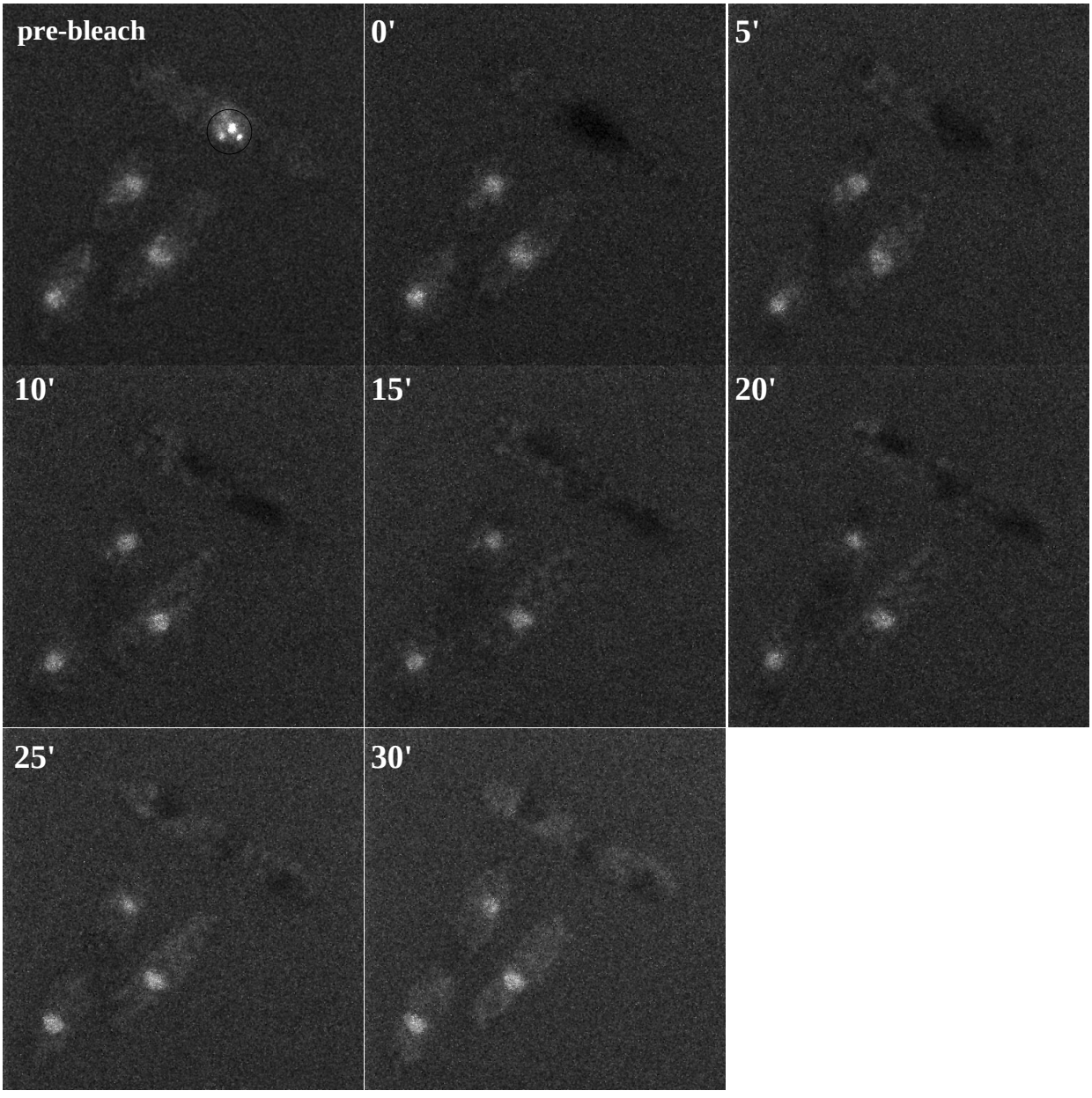

pre-bleach
0'
5'
10'
15'
20'
25'
30'

Figure. 5.9. Bir1p pool on kinetochores moves to the spindle mid-zone in anaphase B. Exponentially growing *gfp-bir1*$^{+}$ *cdc25*-22 cells at 24°C were concentrated by centrifugation. 1µl of cells was spotted on a slide and visualized using the Zeiss LSM510 inverted microscope. The region displaying bright spots of GFP-Bir1p staining was selected as the region of interest (ROI) and subjected to photo bleaching by 100 iterations of the 488 nm He/Ar laser at 100% power. Fluorescence recovery was monitored by time-lapse analysis. Images were captured at 1-minute intervals. Scale bar = 6.0µm.

(Basi *et al*., 1993) have previously been shown to be impaired for sister-chromatid separation, although the cells eventually septate, leading to a '*cut*' phenotype (Funabiki *et al*., 1996). In this study, Cut2Δ80p was generated by deletion of the two destruction boxes located at the N-terminus of Cut2p, which are required for recognition by the anaphase-promoting complex, APC (Funabiki *et al*., 1996). A plasmid (pCDL833) was constructed in which Cut2Δ80p was under control of the weak *nmt1*-81 promoter. pREP81- *cut2Δ80* containing cells, expressing GFP-Bir1p (MBY1493), were grown in the absence of thiamine for 18 hours at 24ºC, fixed and stained to visualize chromosomes, microtubules and GFP-Bir1p. In these cells, chromosomes appeared condensed and were seen clumped to one side of the anaphase spindle. In such cells, it was clearly observed that GFP-Bir1p localized to the mid-zone of anaphase length spindles (Fig. 5.11). This observation suggested that localization of Bir1p to the spindle possibly did not require the loss of cohesion event. The apparent contradiction of results between the above two approaches was resolved in the following experiment.

5.6.2. Cyclin B destruction is required for spindle localization of Bir1p in anaphase B.

dis1Δ gfp-bir1^{+}

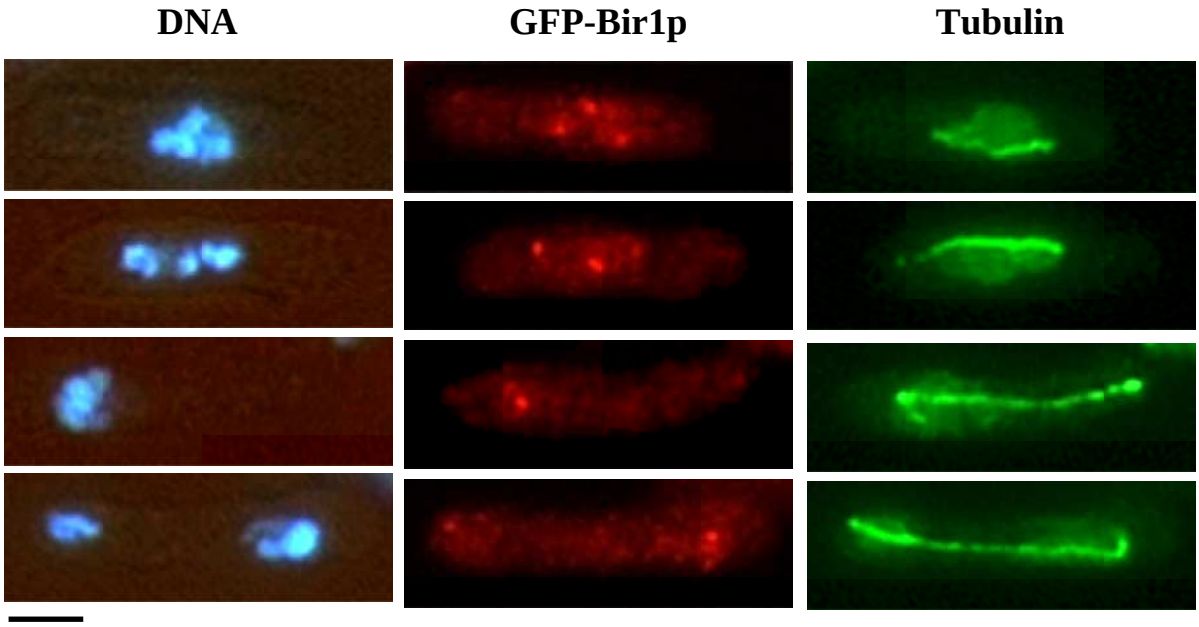


Figure. 5.10. GFP-Bir1p remains on kinetochores in *dis1*Δ cells impaired for loss of sister-chromatid cohesion. Exponentially growing *dis1*Δ::kanR *gfp-bir1*$^{+}$ cells at 32°C were shifted to 19°C for 6 hours. The cells were then fixed with formaldehyde and stained with DAPI to visualize chromosomes, α-GFP to visualize GFP-Bir1p and α-tubulin to visualize microtubules. Scale bar = 3.8μm.

Ubiquitin-mediated cyclin B proteolysis in anaphase leads to the inactivation of the cyclin-dependent kinase (CDK), Cdc2p, which is essential for mitotic exit (Murray and Kirschner, 1989; Glotzer *et al*., 1991). In *S. pombe*, over-expression of truncated cyclin B, Cdc13p, which lacks the 'destruction box' required for recognition by the APC, blocked cells in anaphase with high CDK activity (Yamano *et al*., 1996). Previously, time-lapse analyses have suggested that cyclin B destruction initiates soon after inactivation of the spindle assembly checkpoint (Clute and Pines, 1999). Since temporally, this event closely coincides with the redistribution of Bir1p from kinetochores to the spindle, the dependency of Bir1p localization on cyclin proteolysis was investigated. For this purpose, a strain (MBY1553) expressing GFP-Bir1p was transformed with a plasmid (pCDL834) in which *cdc13*Δ*81* (Cdc13p deleted for its first 81 amino acids) was under control of the medium-strength *nmt1*-41 promoter (Basi *et al*., 1993). Cells were grown in medium lacking thiamine for 18 hours at 24ºC, fixed and stained to visualize DNA, microtubules and GFP-Bir1p. Interestingly, in these cells, GFP-Bir1p localized to more than three kinetochores along the length of a spindle that appeared longer than normal metaphase spindles, but had clearly not initiated anaphase B elongation (Fig. 5.12). The presence of more than three kinetochores indicated that sister-chromatids had undergone disjunction. The distribution of disjoined chromatids along the length of spindles that had not initiated

anaphase B elongation, suggested that pREP41-*cdc13Δ81* cells expressing GFP-Bir1p were probably impaired for completion of anaphase A and subsequent entry into anaphase B.

These observations led to the conclusion that cyclin proteolysis is important for redistribution of Bir1p from kinetochores to the spindle mid-zone.

The conclusions from the above experiment could offer a possible explanation for the apparently contradicting results obtained from the previous experiments on cohesion loss. *dis1*Δ cells arrest with high cyclin B levels and fail to exit from mitosis (Nabeshima *et al*., 1998). It is possible that GFP-Bir1p remains localized to kinetochores in *dis1*Δ cells due to increased cyclin B levels and may not in fact depend on the status of cohesion loss in these cells. Unlike the *dis1*Δ mutant, cells over-expressing Cut2Δ80p are not impaired for cyclin B destruction and perform cell division in the absence of sister-chromatid separation (Funabiki *et al*., 1996). This suggested that the loss of cohesion event that occurs at sister centromeres during metaphase to anaphase transition may not, indeed, be essential for physical relocation of Bir1p from kinetochores to the spindle mid-zone in anaphase.

5.6.3. Microtubules are esssential for the removal of Bir1p from kinetochores

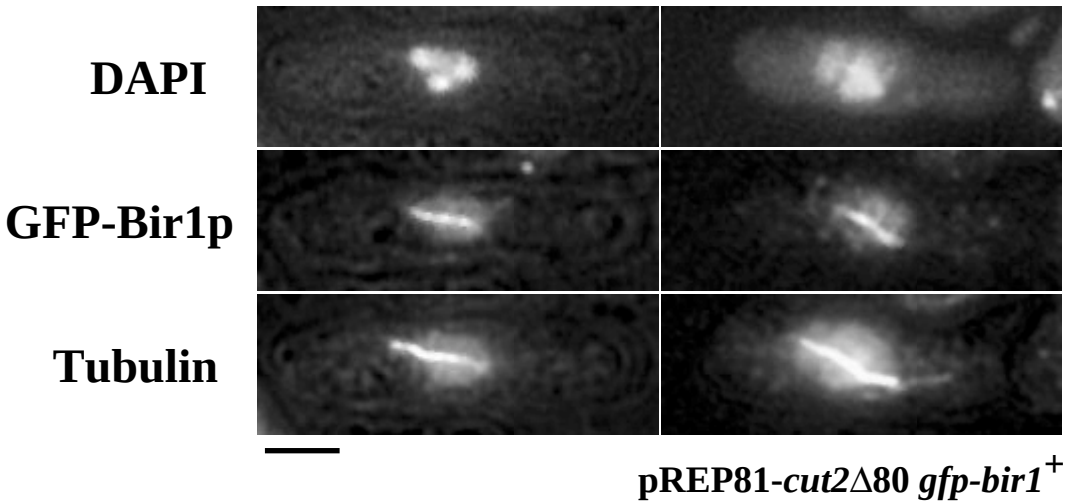

DAPI
GFP-Bir1p
Tubulin
pREP81-*cut2*Δ80 *gfp-bir1*$^{+}$

Figure. 5.11. GFP-Bir1p is located on the spindle mid-zone in cells over-expressing the non-degradable version of Cut2p. Exponentially growing *gfp-bir1*$^{+}$ cells expressing the pREP81-*cut2*Δ80 plasmid in thiamine-containing medium lacking leucine, were washed three times to remove thiamine and re-inoculated in medium lacking both leucine and thaimine. Cells were grown at 24°C for 20 hours, fixed with formaldehyde and stained with DAPI to visualize chromosomes, α-GFP to visualize GFP-Bir1p and α-tubulin to visualize microtubules. Scale bar = 2.8μm.

Since relocalization of Bir1p to the mid-spindle appears to occur only after kinetochores, with the aid of KMTs, reach the spindle poles, it was checked whether MTs were important for the unloading of Bir1p from kinetochores. In order to address this question, a strain (MBY1566) was constructed in which GFP-Bir1p was expressed in the β-tubulin mutant, *nda3*-KM311, which was deleted for the spindle-assembly checkpoint (SAC) gene, *mad2*$^{+}$ (He *et al*., 1997). It has been previously shown that *nda3*-KM311 *mad2*Δ cells (MBY968), when grown at 19°C, fail to arrest at metaphase due to absence of a functional SAC. Hence, the cells proceed through cell division, in the absence of microtubules, owing to destruction of cyclin B (He *et al*., 1997). In this study MBY1566 cells were grown at 19°C for 4 hours, fixed and stained to visualize chromosomes and Bir1p. In these cells, which are devoid of MTs, it was found that GFP-Bir1p remained localized to kinetochores, even in cells that had undergone septation (Fig. 5.13). This observation strongly suggested that the unloading of Bir1p from kinetochores depended on the presence of intact microtubules in the mitotic cell. However, the possibility that Bir1p relocalization to the spindle requires Mad2p function cannot be ruled out. In summary, Bir1p redistributes from kinetochores to the spindle mid-zone at the onset of anaphase B. Although cyclin proteolysis may trigger this event, the presence of intact microtubules is essential for removal of Bir1p from kinetochores in anaphase.

5.7.The dynamics of Bir1p on the mid-zone is independent of spindle microtubule behavior

Anaphase B spindle dynamics has previously been well studied in *S. pombe*. Spindle elongation is proposed to occur by sliding apart of over-lapping microtubules while they polymerize at their plus-ends (Cande and McDonald, 1985; Ding *et al*., 1993; Mallavarapu *et al*., 1999). FRAP analyses have shown that upon metaphase to anaphase transition, an abrupt switch in microtubule dynamics occurs such that anaphase B spindles are much more stable compared to the highly dynamic metaphase spindles (Mallavarapu *et al*., 1999). Photobleaching of the mid-zone region of anaphase microtubules results in minimal fluorescence recovery and the bleached region could be observed to move towards spindle poles indicative of sliding of over-lapping microtubule arrays (Mallavarapu *et al*., 1999). Fig. 5.14 depicts a schematic illustration of this phenomenon. Since the mitotic spindle exhibits such distinct behavior during anaphase B, it was interesting to investigate whether the dynamics of Bir1p on the spindle-midzone coincided with that of MTs.

5.7.1.Minimal turn-over of Bir1p occurs at the spindle mid-zone

FRAP analyses of *cdc25*-22 cells expressing GFP-Bir1p (MBY683), grown at 24°C, were used to visualize Bir1p mid-zone dynamics. Cells exhibiting mid-zone

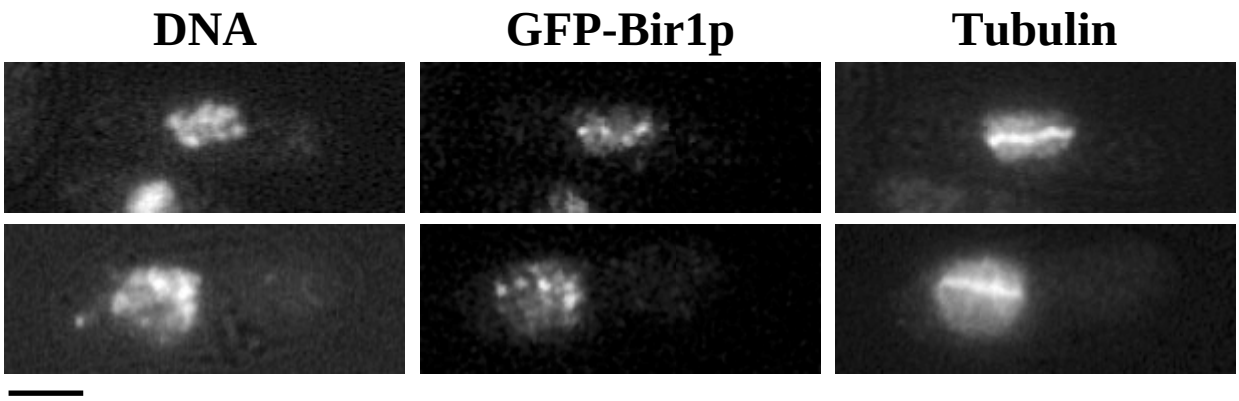

DNA
GFP-Bir1p
Tubulin

Figure. 5.12. Cyclin B proteolysis is required for spindle localization of Bir1p. Exponentially growing *gfp-bir1*$^{+}$ cells expressing the pREP41-*cdc13*Δ81 plasmid in thiamine-containing medium lacking leucine, were washed three times to remove thiamine and re-inoculated in medium lacking both leucine and thiamine. Cells were grown at 24°C for 20 hours, fixed with formaldehyde and stained with DAPI to visualize chromosomes, α-GFP to visualize GFP-Bir1p and α-tubulin to visualize microtubules. Scale bar = 3.2μm.

localization of GFP-Bir1p were chosen for bleach analysis (n=7 cells). A series of images from a single FRAP experiment are shown in Fig. 5.15. The entire mid-zone fluorescence of GFP-Bir1p was abrogated upon photo bleaching with laser conditions as described before (Fig. 5.15 time point 0'). After 4 minutes, it was observed that GFP-Bir1p failed to recover any detectable fluorescence at the mid-zone (Fig. 5.15 time points 1'0'' to 4'0''). The fluorescence did not recover to its original level even after a 15 -minute post-bleach interval (data not shown). This result indicated that Bir1p undergoes minimal turn-over at the spindle mid-zone.

5.7.2. Bir1p protein sub-units undergo fluorescence recovery within the spindle mid-zone.

It has so far been suggested that the Bir1p on the spindle mid-zone, after redistribution from kinetochores, undergoes minimal turnover during the course of anaphase B. Since active tubulin polymerization occurs at MT plus-ends, it is interesting to speculate on the factors that retain Bir1p at over-lapping ends of the anaphase B spindle. In order to obtain further clues into this mechanism, fluorescence recovery was analyzed in cells in which only a section of GFP-Bir1p-stained mid-zone was photo-bleached. A series of images from FRAP experiments in which different sections of the mid-zone were bleached are shown in Fig. 5.16 A to C. Upon

bleaching the middle section (Fig. 5.16 A; n=8 cells), or one end of the mid-zone (Fig. 5.16 B; n=5 cells), fluorescence recovery of GFP-Bir1p was observed after an interval of about one minute. It appeared as though the GFP-fluorescence redistributed from the brightly stained mid-zone region (Fig. 5.16A time point 2'30'' and Fig. 5.16B time point 1'30''). Bleaching of both the ends (Fig. 5.16 C; n=3 cells) also resulted in detectable recovery of fluorescence after one minute. In both B and C, it was observed that the intensity of recovered fluorescence was significantly lower than the intensity prior to bleach (Fig. 5.16D). Since no turnover of Bir1p occurred when the entire mid-zone region was bleached, it can be suggested that the fluorescence recovery that is observed in three different conditions tested above may occur due to two possible reasons. Sliding of overlapping MTs away from each other may cause apparent recovery of fluorescence in the bleached region (refer to scheme in Fig. 5.18A). Alternatively, recovery may occur due to lateral re-distribution of the remaining unbleached protein on the mid-zone. In both scenarios, the resultant fluorescence intensity would not match up to pre-bleach levels.

In summary, it can be concluded that Bir1p undergoes minimal flux when localized at the spindle mid-zone. Its behavior on the anaphase spindle is different from tubulin dynamics. Interestingly, redistribution of Bir1p seems to occur within

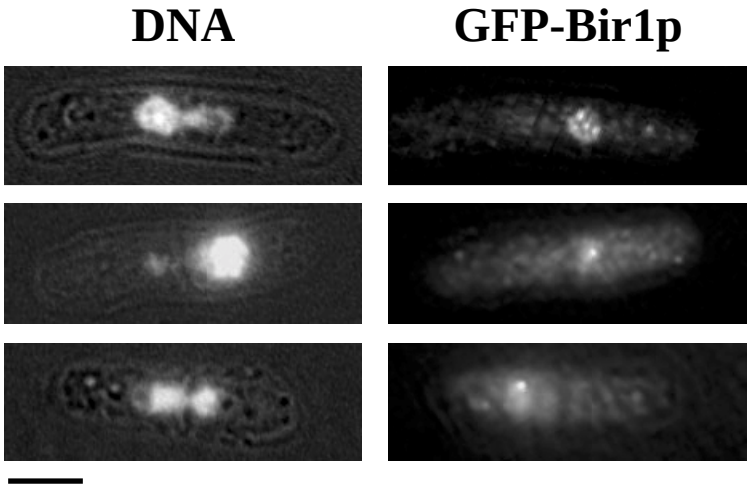
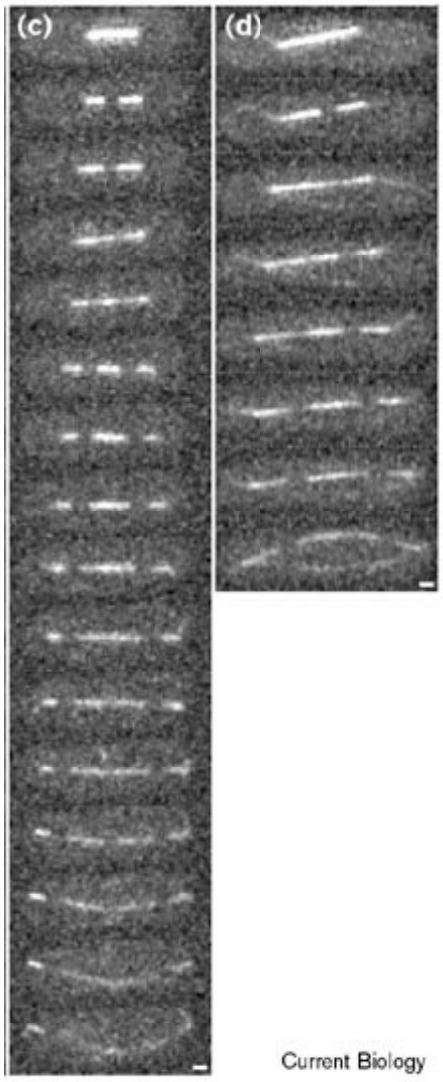

DNA
GFP-Bir1p

Figure. 5.13. Microtubules are essential for removal of Bir1p from kinetochores. Exponentially growing *nda3*-KM311 *mad2*Δ cells expressing GFP-Bir1p at 32°C were shifted to 19°C for 4 hours. Cells were then fixed with formaldehyde and stained with DAPI to visualize chromosomes and α-GFP to visualize GFP-Bir1p. Scale bar = 4.1μm.

the mid-zone, possibly due to lateral movement of the protein within the mid-zone region, or alternatively fluorescence recovery may occur simply due to the sliding motion of MT arrays.

5.8. Maintenance of Bir1p on the spindle mid-zone requires microtubules.

Since the dynamics of Bir1p on the spindle mid-zone differed from that of tubulin sub-units, it can be asked whether the presence of microtubules was essential for maintenance of Bir1p at the mid-zone. In order to address this issue, *cdc25*-22 cells expressing GFP-Bir1p (MBY683) were synchronously released into mitosis at 24°C from G2 arrest. Around 45 minutes after release, one half of the culture was fixed and stained with GFP and tubulin antibodies as control. The other half of the culture was subjected to an ice-water bath treatment for 30 minutes prior to fixation. It has been previously shown that cold-shock treatment of *S. pombe* cells causes complete microtubule de-polymerization (Tran *et al*., 2001). The control culture showed a high proportion of cells in anaphase B with GFP-Bir1p staining the mid-zone of elongated spindles (Fig. 5.17A). This affirmed that the other half of the culture contained an enriched population of anaphase B cells with GFP-Bir1p on the spindle mid-zone prior to microtubule de-polymerization upon cold-shock. Tubulin and GFP staining revealed that in a vast majority of anaphase B cells, GFP-Bir1p



Adapted from Mallavarapu et al 1999

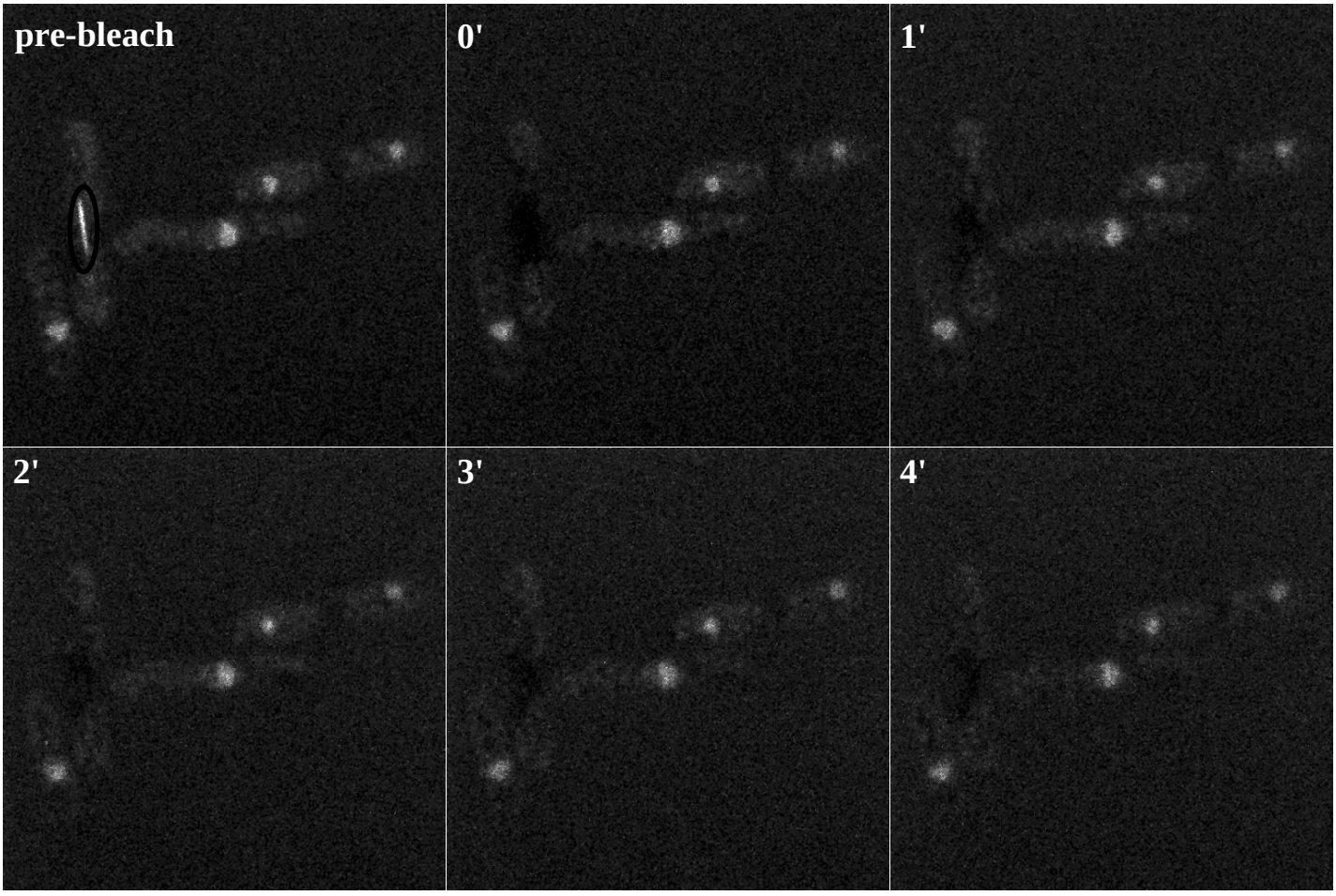
Figure. 5.14. Schematic illustration of the results of the FRAP analyses of microtubules during anaphase B, performed by Mallavarapu *et al*., 1999. Photo bleaching of the mid-region of the anaphase B spindle resulted in the splitting of the bleached region into two parts which moved away from each other towards opposite spindle poles. This movement indicated that the anaphase B microtubules were stable and the spindle elongated by sliding of over-lapping arrays of MTs.

localization to the spindle mid-zone was completely abolished upon complete microtubule de-polymerization, and only general nuclear staining of the protein was observed (Fig. 5.17B). It can be concluded that in spite of the unique dynamics exhibited by Bir1p at the spindle mid-zone, maintenance of this protein on the mid-zone requires the presence of a structurally intact anaphase B spindle.

5.9.Discussion

5.9.1. Cellular localization of Bir1p

This study has analyzed the cellular localization pattern of the *S. pombe* Survivin homolog, Bir1p. In interphase cells, Bir1p localizes to multiple nuclear dots, one of which represents clustered-centromeres at the spindle pole body (SPB). Bir1p appears to remain localized at centromeres until its abrupt redistribution from kinetochores to the mid-zone of the anaphase spindle. The mitotic localization pattern of Bir1p suggests that this protein operates as a *bona fide* chromosome passenger in *S.pombe*. Interestingly, an identical pattern of Bir1p localization to kinetochores and the spindle mid-zone was observed in meiotic cells, which undergo both reductional and equational nuclear divisions. This suggests that Bir1p may play a fundamental role in the process of chromosome segregation. It was observed that upon localization to the spindle, Bir1p was no longer detectable at kinetochores through the course of


pre-bleach
0'
1'
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4'

Figure. 5.15. Bir1p exhibits minimal turnover on the spindle mid-zone. Exponentially growing *gfp-bir1*$^{+}$ *cdc25*-22 cells at 24°C were concentrated by centrifugation. 1µl of cells was spotted on a slide and visualized using the Zeiss LSM510 inverted microscope. The region displaying bright mid-zone staining of GFP-Bir1p staining was selected as the region of interest (ROI) and subjected to photo bleaching by 100 iterations of the 488 nm He/Ar laser at 100% power. Fluorescence recovery was monitored by time-lapse analysis. Images were captured at 1-minute intervals. Scale bar = 7.0µm.

mitosis. Since Bir1p is detected on centromeres in interphase (both in S-phase and G2), it will be interesting to analyze the factors that regulate reloading of this protein onto chromosomes after exit from mitosis. The study by Morishita *et al* (2001) has shown that kinetochore-localization of Bir1p is lost in cohesin mutants, *rad21* and *mis4*. Future studies should check whether centromeric loading of Bir1p is dependent on the establishment of sister chromatid cohesion at S-phase

5.9.2. Temporal regulation of Bir1p localization in mitosis

Previous studies, using the FISH technique, have documented anaphase movement of kinetochores in *S. pombe*. It was shown that mitotic kinetochores congress to a bright spot at the spindle equator, indicative of a metaphase plate-like structure. Upon loss of cohesion, kinetochores move in a directional manner towards opposite spindle poles in anaphase A (Funabiki *et al*., 1993). Similar dynamics exhibited by Bir1p-stained kinetochores in this study has revealed that Bir1p remains localized to kinetochores until chromosomes reach the spindle poles at the end of anaphase A.

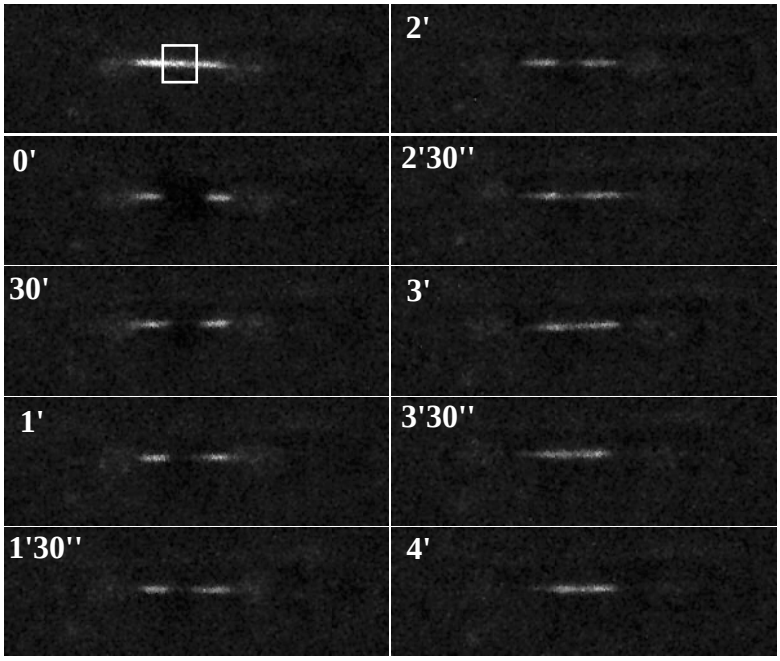
This idea is further supported by kinetochore localization of GFP-Bir1p in *klp5*Δ mutants that are impaired for anaphase A. The plus-end directed kinesin, Klp5p, is thought to be important for efficient execution of anaphase A by promoting

kinetochore MT disassembly (West *et al*., 2001; West *et al*., 2002). Perhaps, Bir1p redistribution to the spindle can occur only when kinetochores physically reach the spindle poles at the end of anaphase A. The observation that the same protein pool of Bir1p moves from kinetochores to the spindle mid-zone, which has been suggested by FRAP analyses in this study, further lends support to this hypothesis. It is tempting to speculate that redistribution of Bir1p, possibly as part of the chromosome passenger complex, from kinetochores to the spindle may serve to coordinate the completion of anaphase A with the onset of anaphase B.

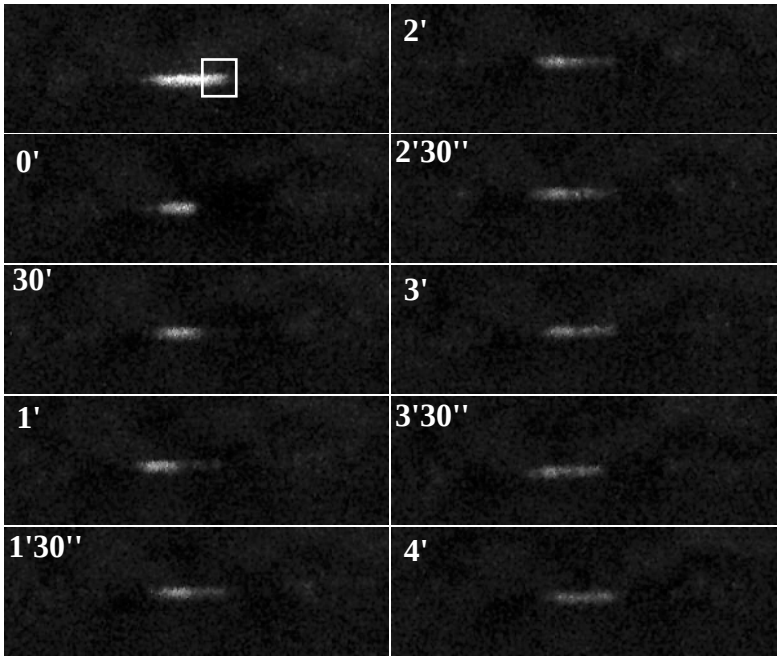
5.9.3. Factors that regulate kinetochore to spindle relocation of Bir1p.

This study has shown that Bir1p movement from kinetochores to the mid-zone is dependent on mitotic destruction of cyclin B. A similar regulation by CDK has been demonstrated for aurora B dynamics in mammalian cells (Murata-Hori *et al*., 2002). How does cyclin proteolysis trigger this abrupt transfer of Bir1p from one structure to the other? One possibility is that the trigger is regulated by phosphorylation of Bir1p by the cyclin dependent kinase. In fact, Bir1p contains seven potential Cdc2p phosphorylation sites. Cyclin destruction may result in dephosphorylation of Bir1p causing its redistribution to the spindle. Interestingly, it was recently shown in *S. cerevisiae* that the conserved phosphatase Cdc14p, which is

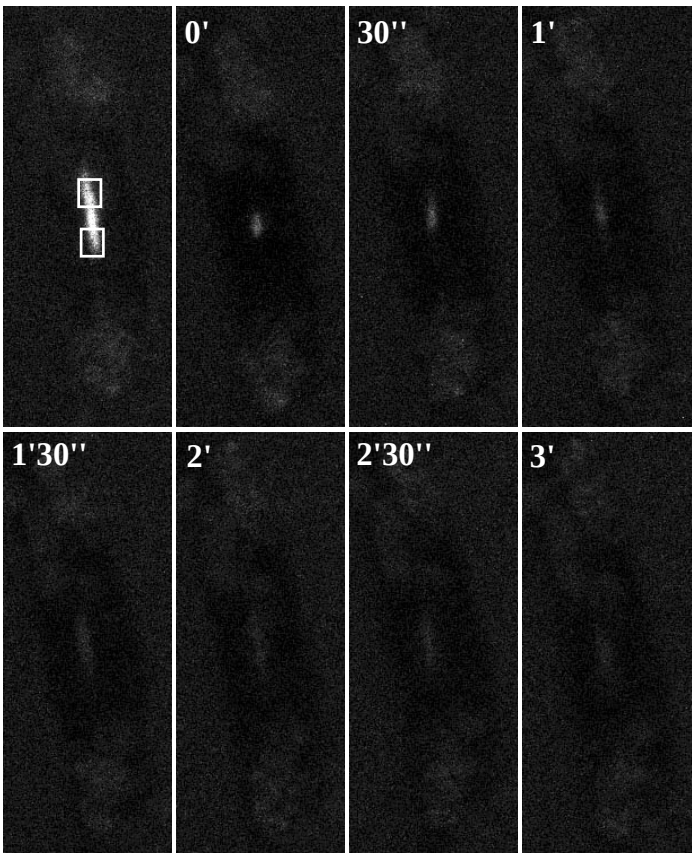
A)



B)



C)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1'
1'30''
2'
2'30''
3'

D)

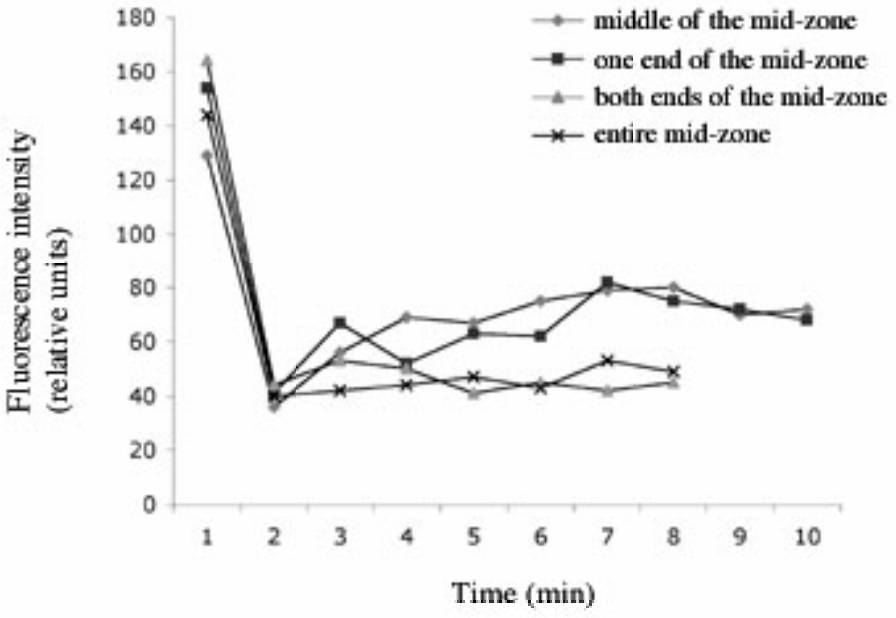
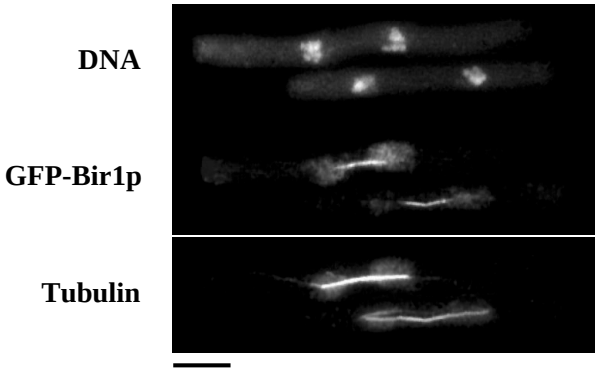

middle of the mid-zone
one end of the mid-zone
both ends of the mid-zone
entire mid-zone
Fluorescence intensity
(relative units)
180
160
140
120
100
80
60
40
20
0
1
2
3
4
5
6
7
8
9
10
Time (min)

Figure. 5.16. Dynamics of Bir1p within the spindle mid-zone. Exponentially growing *gfp-bir1*$^{+}$ *cdc25*-22 cells at 24°C were concentrated by centrifugation. 1µl of cells was spotted on a slide and visualized using the Zeiss LSM510 inverted microscope. The middle (A), one end of (B) and both ends (C) of the region displaying bright mid-zone staining of GFP-Bir1p staining was selected as the region of interest (ROI) and subjected to photo bleaching by 100 iterations of the 488 nm He/Ar laser at 100% power. Fluorescence recovery was monitored by time-lapse analysis. Images were captured at 30-second intervals. D) Graphical representation of fluorescence recovery upon photo-bleaching.

important for regulation of mitotic exit (Vinstin *et al*., 1998), regulates localization of the INCENP-Aurora B complex to the spindle by dephosphorylating the budding yeast INCENP homolog, Sli15p (Pereira and Schiebel, 2003).

Alternatively, cyclin proteolysis may promote sister chromatid movement to the spindle poles in anaphase A, thereby aiding physical redistribution of Bir1p to the spindle in anaphase B. This study has shown that cells expressing non-degradable cyclin B are possibly impaired in completion of anaphase A. Recent evidence in *Drosophila* embryos suggests that anaphase A kinetochore movement required cyclin B proteolysis (Parry *et al*., 2003). However, the mechanism of Bir1p relocation may not be that simple, as this study has shown that Bir1p moves to the spindle-midzone in spite of the presence of non-disjoined sister-chromatids. Additionally cyclin proteolysis may not be sufficient to trigger unloading of Bir1p from kinetochores. In this study, it was shown that Bir1p fails to dislodge from kinetochores in the absence of microtubules, even though cyclin proteolysis and mitotic exit occurred. Perhaps microtubules physically aid the disembarkation of Bir1p from kinetochores, possibly via the aid of motors. Altogether, it will prove interesting to further analyze the mechanisms that regulate movement of Bir1p from one mitotic structure to the other.

A)



B)

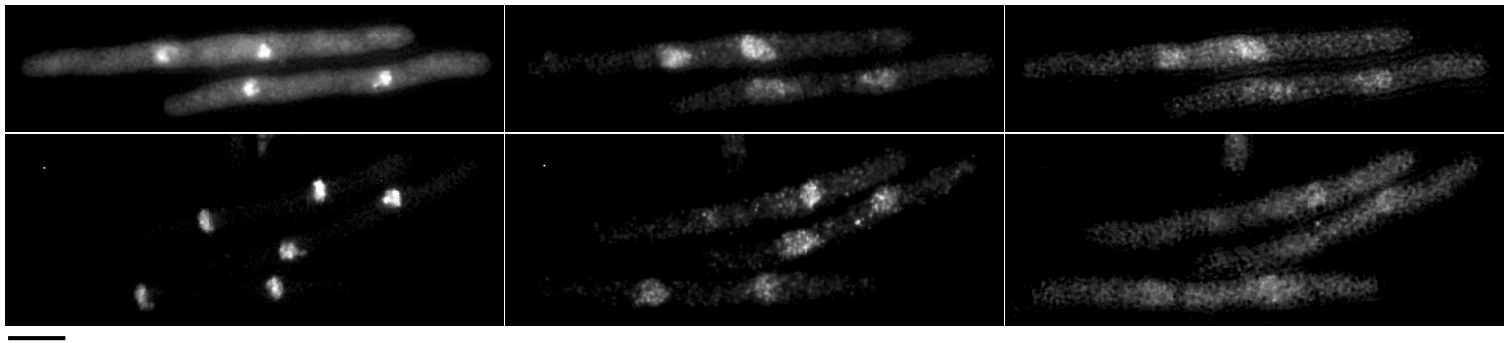


Figure. 5.17. Maintenance of Bir1p on the mid-zone requires the presence of an intact anaphase B spindle. A) Exponentially growing *gfp-bir1*$^{+}$ *cdc25*-22 cells at 24°C were shifted to 36°C for 4 hours. The cells were synchronously released into mitosis at 24C for 45 minutes, fixed with formaldehyde and stained with DAPI to visualize chromosomes, α-GFP to visualize GFP-Bir1p and α-tubulin to visualize microtubules. B) Exponentially growing *gfp-bir1*$^{+}$ *cdc25*-22 cells at 24°C were shifted to 36°C for 4 hours. The cells were synchronously released into mitosis at 24°C for 45 minutes and then subjected to an ice-water bath treatment for 30 minutes. The cells were then fixed with formaldehyde and stained with DAPI to visualize chromosomes, α-GFP to visualize GFP-Bir1p and α-tubulin to visualize microtubules. Scale bar = 5.5μm.

5.9.4. Dynamics of Bir1p on the spindle mid-zone

Anaphase B spindle elongation is thought to be a result of sliding apart of the overlapping arrays of microtubules combined with continuous polymerization of tubulin at their plus ends (Cande and MacDonald, 1985; Ding *et al*., 1993). In *S. pombe* it has been previously shown that photobleaching of the anaphase B mid-spindle results in the bleached zone splitting into two parts which move away from each other towards the poles. This movement was indicative of the sliding motion of overlapping MT arrays away from each other (Mallavarapu *et al*., 1999). In this study, when a small region in the middle of Bir1p-stained mid-zone was bleached, fluorescence recovery occurred. Similar dynamics of Bir1p was observed when one end or both ends of the mid-zone region were bleached, although a net decrease in recovered fluorescence levels was observed. Interestingly, bleaching the entire mid-zone region failed to show any detectable recovery of Bir1p fluorescence. Hence, it is appears that the behavior of Bir1p on the spindle mid-zone differs from that of tubulin sub-units, though its maintenance at the mid-zone still depends on the presence of an intact anaphase B spindle. Based on the results from the mid-zone bleach experiments, it is possible that apparent recovery of fluorescence may be detected due to the sliding motion of anti-parallel MT arrays (refer to scheme in Fig. 5.18A). Alternatively, since no new addition of Bir1p occurs on the spindle, the recovery of

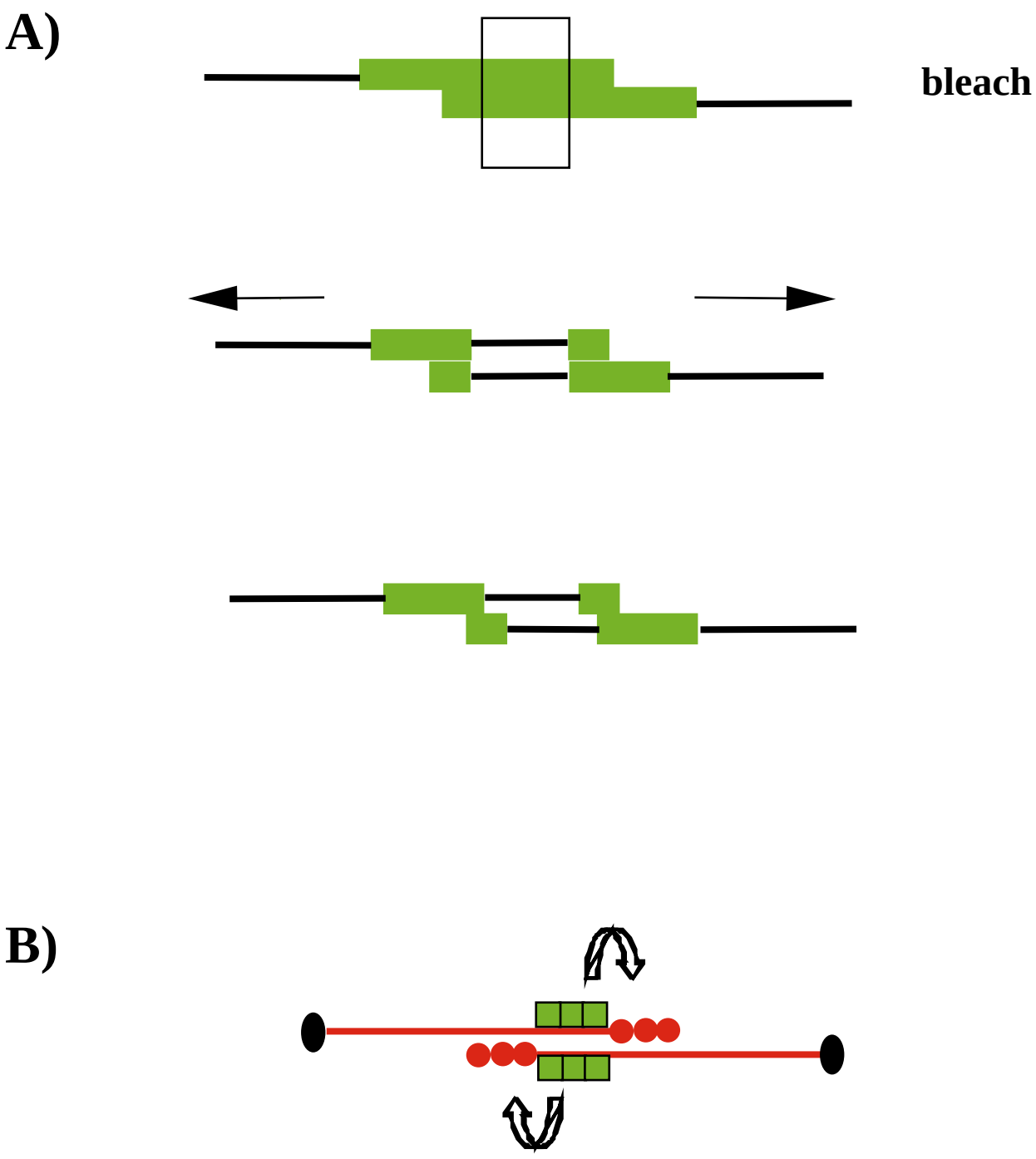

A)
bleach
B)
SPB
Tubulin subunit
Tubulin
Bir1p

Figure. 5.18. Schematic illustrations of the possible mechanisms of Bir1p redistribution within the spindle mid-zone. A) Sliding motion of MTs causes GFP-Bir1p to distribute within the mid-zone region upon photo bleaching. B) Bir1p redistribution may occur to new binding sites on incoming tubulin subunits at the plus-ends.

fluorescence may be a consequence of lateral redistribution of Bir1p molecules within the mid-zone.

The observation that Bir1p does not recover fluorescence when the entire spindle mid-zone is bleached suggests minimal turnover of the protein at this site. A less interesting possibility is that at the steady state, a vast majority of the total protein in the cell is at these sites, thus when it is bleached, there is no unbleached protein to replace it. However, what retains Bir1p at the spindle mid-zone? FRAP analyses in this study have shown that Bir1p does not appear to get added on to plus-ends along with new incoming tubulin sub-units from the nucleoplasm. It is possible that Bir1p molecules on the mid-zone could simply be redistributing to new binding sites available on polymerized tubulin at the plus-ends (refer to scheme in Fig. 5.18B). Alternatively, Bir1p could be retained at the mid-zone as a consequence of its binding to plus-end motors. In future, FRAP analyses of mid-zone motor proteins should provide further insight into the similarities/differences between the dynamics of motors and the dynamics of passengers.

CHAPTER 6 – Discussion

The process of accurate chromosome segregation lies at the heart of cellular reproduction. A combination of chromosomal and cytoskeletal events occur in a coordinated manner to initiate and complete the process of mitosis in order to produce viable progeny.

The Aurora B-Survivin-INCENP complex of proteins forms a group of 'chromosomal passengers' that move from kinetochores in metaphase to the mid-spindle in anaphase. In higher organisms, this complex eventually localizes to the mid-body and the division furrow (Carmena and Earnshaw, 2003). This striking localization pattern of these proteins through the course of mitosis has led to the idea that the passenger complex may perform essential functions at different cellular locations to coordinate mitotic and cytokinetic events.

My study describes the analysis of the role of *S. pombe* Bir1p in mitosis. Bir1p is a nuclear protein that physically relocates from kinetochores to the spindle mid-zone at anaphase A to anaphase B transition. This protein is important for multiple processes in mitosis including chromosome condensation and spindle elongation. It can be speculated that the various mitotic functions of Bir1p are dictated by its cellular location at different stages in mitosis. At the kinetochore,

Bir1p is essential for maintaining mitotic chromosome architecture possibly via recruitment of the Aurora B kinase, Ark1p. Aurora B kinase is thought to be important for chromosome compaction through phosphorylation of the serine 10 residue on histone H3, which has been proposed to signal the loading of the Condensin machinery onto chromosomes (Wei *et al* 1999; Hsu *et al* 2000; Giet and Glover 2001). In future, it will be interesting to investigate the mechanisms by which Bir1p and Ark1p, resident at kinetochores, influence the status of condensation on the arms of chromosomes.

Time-lapse analyses have shown that Bir1p remains on kinetochores until the end of anaphase A, when sister-chromatids reach the spindle poles, after which it relocates to the spindle mid-zone. Could the physical movement of this protein from kinetochores to the spindle serve as a signal to co-ordinate anaphase A and B? Interestingly, this study has shown that *bir1* mutants are defective in anaphase chromosome movement, and appear to initiate anaphase B spindle elongation without completing anaphase A. Further credence to this hypothesis is lent by the observation that Bir1p movement to the spindle is under regulation of cyclin B proteolysis. This regulation could be a means to ensure that the passenger complex gets loaded on to the spindle only after the separated sister-chromatids reach the poles, thereby coordinating chromosomal events with cytoskeletal events.

Upon relocation to the spindle, Bir1p is found restricted to the mid-zone throughout anaphase B. What are the possible functions of Bir1p (and the passenger complex) on the spindle mid-zone? This study has demonstrated that lack of Bir1p prevents complete elongation of the anaphase B spindle. Owing to its localization to the mid-zone, the region of MT overlap, it is possible that Bir1p stabilizes the spindle during elongation. This may be achieved by recruitment of bundling proteins or plus-end motors that cross-link microtubules and generate the force needed to elongate the spindle. Alternatively, Bir1p may itself structurally bind over-lapping MT arrays. Recent crystal structure studies have shown that the human Survivin forms a unique bow-tie shaped dimer (Chantalat *et al*., 2000). Perhaps, dimerization between Bir1p molecules on over-lapping arrays of MTs may physically hold the spindle together. Time-lapse analyses of mitotic spindle elongation and detailed analyses of the nature of the spindle mid-zone in *bir1* mutant cells should provide further insight into the function of Bir1p on the spindle.

Although much is known about the general structure and physics of the bipolar spindle, it is not clear what defines and constitutes the mid-zone. The mid-zone differs from the rest of the spindle owing to the presence of over-lapping MTs, which possibly defines its properties. My study has shown that Bir1p behavior on the spindle mid-zone is different from that of tubulin sub-units. Whether this behavior is

unique to Bir1p or do other mid-zone proteins display similar dynamics should be a subject for future studies. It can be speculated that Bir1p continuously redistributes to binding sites available on the newly polymerized tubulin subunits at plus ends, and thus remains localized at the mid-zone. However, the question of what restricts the protein from spreading along the length of the entire spindle still remains unanswered. Future studies should address the factors that are involved in defining spindle mid-zone boundaries. Isolation of mutants that fail to restrict Bir1p (and other mid-zone proteins) to the spindle mid-zone might provide a starting point to address this issue.

Concluding remarks

This study has provided a framework for the understanding of the function of Bir1p in mitosis. Future studies should help unravel the precise mechanisms by which Bir1p influences chromosome condensation and spindle integrity and function. Of particular interest also is the role of the spindle mid-zone in mitosis. By offering a simplistic yet conserved view of the mitotic apparatus, the fission yeast may prove a useful system to address all the intriguing questions posed above, in the years to come.

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